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QUANTITATIVE STUDIES OF THE FACTORS GOVERNING THE RATE OF REGENERATION IN TUBULARIA

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The purpose of these investigations is to determine the reciprocal effect of two regenerating regions in *Tubularia*. In order properly to design the experiment it is necessary first to work out the rates of regeneration of different parts of the stem and to determine the effect of size of stem on rate of regeneration. Following this two regions of known rates are allowed to compete with one another and the effect on rate of regeneration is measured. Since it is found that the region exhibiting the lower rate is most affected, further experiments are designed to measure this effect. This inhibitory effect exercised by the higher rate over the lower rate has been termed physiological dominance by Child (1929) and the term "dominance" is used in this paper to describe inhibition.

When the stem of *Tubularia* is sectioned the hydranth differentiates *in situ* from the tissue of the cut end and after 30–40 hours at 18° C. the length of the primordium can be measured by means of an ocular micrometer. At this time a constriction appears at the base of the developing hydranth separating it from the rest of the stem. The perisarc being rigid, the diameter of the hydranth can be measured and the volume calculated. The time for regeneration is measured at two stages of development. t_1 is the time in hours from the time at which the stem is cut to the time at which the constriction appears between the primordium and the rest of the stem. t_2 is the time for complete regeneration when the hydranth is pushed out of the opening of the perisarc. In some experiments where short pieces of stem are used it is necessary to use t_1 as a measure of time since the hydranth, although completely differentiated, does not emerge from the end. In most cases, however, t_2 is used for the calculation of rate of regeneration.

Rate of regeneration can then be defined as the volume of the hydranth in cubic micra divided by the time in hours. $R = \pi r^2 L/t$.

Naturally when t_1 is used the rate is somewhat higher than when t_2 is the measure of time. The values obtained are in regeneration units which are termed R. U. in this paper.

It is convenient to assign symbols to the ends and this has been done in Fig. 1 which shows the method of naming the hydranths. D_1, D_2, D_3, etc. are used to designate the distal or oral hydranth of each piece of the stem and are numbered consecutively from distal to

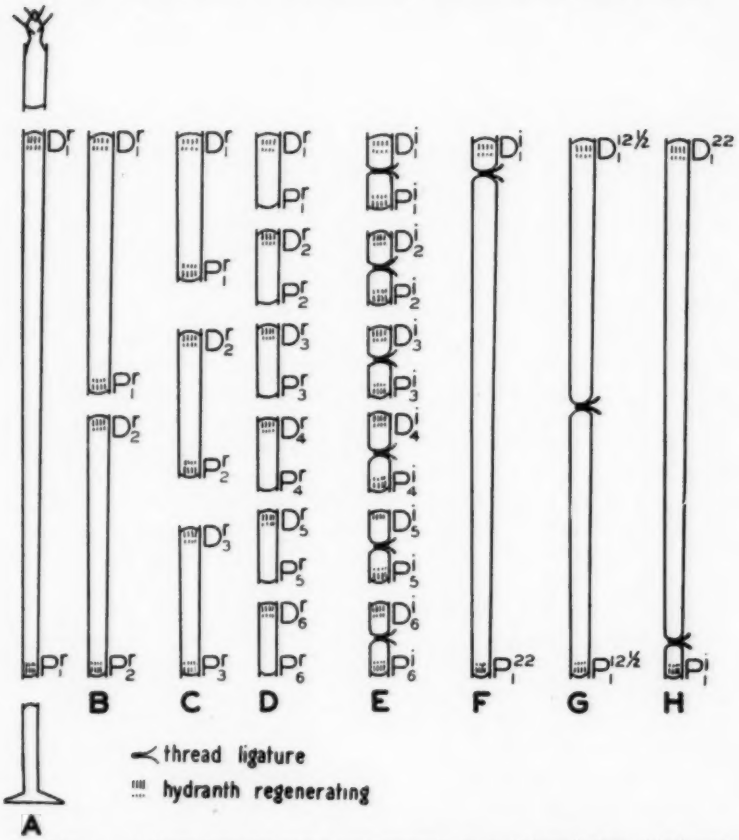

FIG. 1. *A* shows how the stems are prepared; *B*, *C*, *D* how halves, thirds and sixths are prepared and designated; *E* shows how inherent rates are determined by tying off a small part of the stem; finally, *F*, *G*, *H* show how the length of stem is varied while the level remains constant. $D_1^r, D_2^r \cdots$ relative rate of distal hydranths; $P_1^r, P_2^r \cdots$ relative rate of proximal hydranths; $D_1^i, D_2^i \cdots$ inherent rate of distal hydranths; $P_1^i, P_2^i \cdots$ inherent rate of proximal hydranths. D^m and P^m are examples of absolute rates.

proximal levels. D_1 is always the most distal hydranth of the stem. P_1, P_2, P_3, etc. are used for the proximal or aboral hydranths in the same order. A glance at Fig. 1, *A*, *B*, *C*, will make this clear.

The stems are kept in a rectangular dish which is cooled by a glass coil through which running sea water is circulated. The stems rest on a strip of cheesecloth which is stretched over a glass frame and are covered by one centimeter of sea water. Loose-fitting glass covers are placed over the top of the dish. A glass rod, which is drawn out and bent in the form of a hook, is used for picking up and transferring long stems. For short stems a pipette bent at a 45° angle is satisfactory.

Since the stems of *Tubularia* are very variable in the rate at which they regenerate, controls were prepared for every experiment, and experiments involving the use of different stems are never compared. That is to say, one may not compare the rates of 15-mm. stems from different experiments for they may be vastly different, as measurements show (Table II, Experiments 5, 6 and 7).

The general procedure was to select from several colonies those colonies with straight stems and no side branches. The stems were cut off at the base and placed in a large dish of sea water and further selected for similarity in length, diameter and appearance. Some stems were translucent, while others were opaque, and these two differed in rate of regeneration. After this selection, the hydranths were cut off, and the stems of the same length were selected at random for experimentals and controls. In long stems, the additional precaution of cutting off 3 mm. of the stem along with the hydranth was observed, as the region adjacent to the hydranth often regenerates at a low rate, due possibly to the use of this region in the formation of the very large gonophores.

The Inherent Rates of Regeneration at Various Regions of the Stem

The object of these experiments is to isolate various levels of the stem so that the inherent rate of regeneration can be determined. Isolation of a region from the rest of the stem can be obtained by means of a thread ligature which is tied about the stem shutting off circulation and cutting through the tissue. This technique was used by Morgan (1902) and lately by Peebles (1931). The perisarc, which is tough, does not crack but the coenosarc is completely severed so that there are no cellular connections across the ligature. If this ligature be applied about 2–3 mm. from the end of the stem a small piece of tissue 2–3 mm. in length is isolated and will form a hydranth. By this means the rate of regeneration of a small piece of tissue at any level

of the stem can be measured without being influenced by the stem as a whole. The situation is analogous to self-differentiation of an explant from an embryo. It might be thought that the same result could be brought about more simply by cutting off a piece 2–3 mm. in length. However, in this case one meets with the difficulty that two centers of regeneration arise at the two cut ends and bipolar forms may arise. It is impossible to get any measure of the rate at which these forms develop.

TABLE I

Inherent rates of regeneration of distal and proximal hydranths at various levels of the stem of *Tubularia*. Stems 30 mm. long, cut into 6 pieces and each piece ligatured in middle. L = length in μ, d = diameter of stem in μ, t_1 = time in hours from cutting to the constriction of the regenerating hydranth.

	L	d	t_1	$\frac{L}{t_1}$	$\frac{\pi r^2 L}{t_1} \cdot 10^6$
D_1	1136	504	31.6	36.0	71.6
D_2	1044	488	34.4	30.3	56.4
D_3	928	456	36.4	26.2	43.3
D_4	880	432	39.6	22.2	33.1
D_5	592	388	39.8	14.8	17.6
D_6	404	364	49.1	8.2	8.7
P_1	924	504	39.1	23.6	47.0
P_2	824	456	39.3	21.0	33.9
P_3	844	432	40.9	20.6	30.3
P_4	704	408	47.5	14.8	19.4
P_5	416	372	44.5	9.2	9.6
P_6	244	340	52.8	4.6	4.2

The method of determining the inherent rates of regeneration at various levels of the stem is shown in Fig. 1, E, where the superscript "i" is used to designate inherent rate. Stems 30 mm. in length are first cut into 6 pieces and then each 5-mm. piece is ligatured in the middle. Thus in each 5-mm. stem the distal half is completely isolated from the proximal half. Since the stem which regenerates is so short, 2.5 mm., the hydranths have difficulty in emerging and so the time recorded is t_1. This is the time from cutting to the formation of the primordium of the hydranth.

In Table I and Fig. 2 the data are recorded and plotted. The rates are calculated in two ways. $R_1 = \frac{L}{t_1}$ and also $R_2 = \pi r^2 L/t_1$ where L = length of primordium in micra, r = radius of stem in micra and t_1 = time in hours. From the table it is seen that there is a gradient in the size of the hydranth and the time for regeneration. The rate

of regeneration R_2 of the distal hydranths $D_1 - D_6$ thus falls off sharply from $D_1^i = 71.6$ R. U. at the distal end to $D_6^i = 8.7$ R. U. at 5 mm. The lowest rate at 0 mm. is $P_6^i = 4.2$ R. U. Thus the most distal level of the stem regenerates at almost 18 times the rate of the most

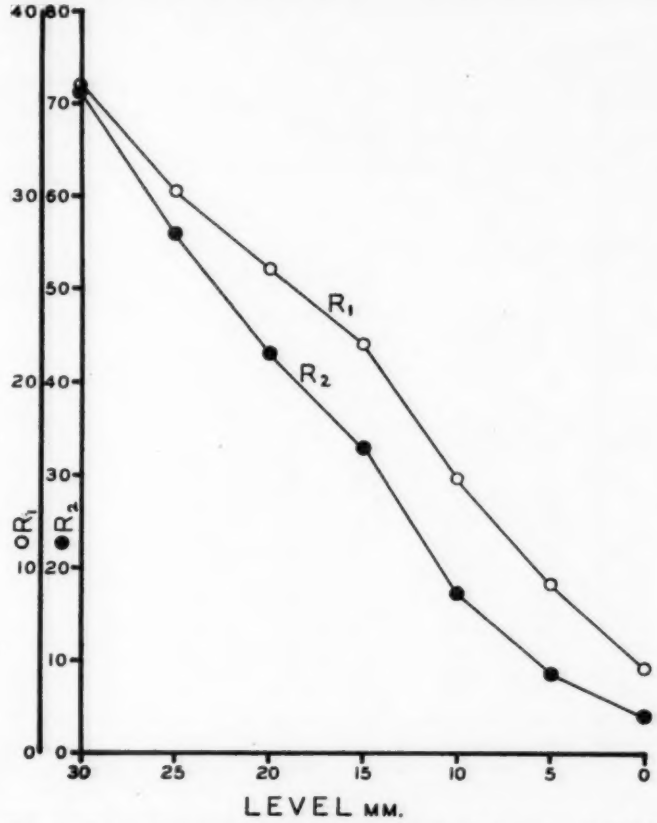

FIG. 2. Rates of regeneration at different levels of the stem of *Tubularia*. $R_1 = \frac{L}{t_1}$; $R_2 = \pi r^2 L/t_1 = \mu^3 \cdot 10^3$/hours. Stems 30 mm. in length cut into 6 pieces and ligatured in middle. Each point is the average of 10 stems. r = radius of cross-section of stem; L = length of primordium; t_1 = time in hours between cutting and the formation of the primordium. The lowest rate is rate of P_6. Zero mm. is at the proximal end of the stem, 30 mm. at the distal end.

proximal level. This difference in rate must be due to causes inherent in the tissue at these levels since external influences have been removed.

The rate of regeneration R_1 has also been calculated using length as a measure of the size of the primordium in place of volume. These calculations are included here to show that the difference in rate of the regenerating hydranths at various levels of the stem is not due merely to a difference in the diameter of the stem but also to a difference in

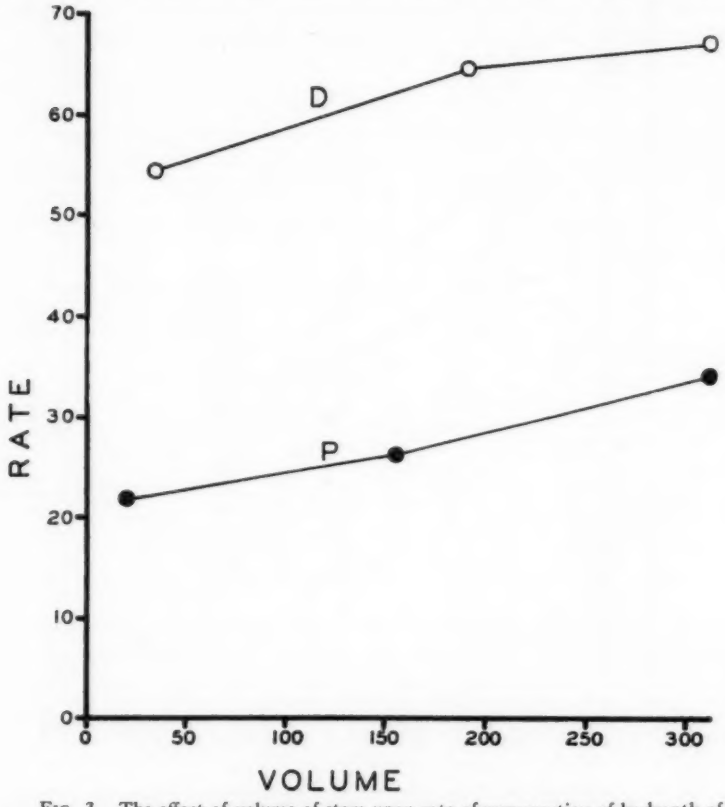

FIG. 3. The effect of volume of stem upon rate of regeneration of hydranth of *Tubularia*. D = distal hydranth; P = proximal. Rate = μ^3/hrs.$\cdot 10^5$. Volume = $\mu^3 \cdot 10^7$. Time in this experiment is t_2.

the length of the primordium. What R_1 really represents is the rate of regeneration R_2 divided by the cross-sectional area of the stem and therefore it corrects for the variation in diameter caused by the growth form of the stem. For obviously if the stem is always wider at the distal end as compared with the proximal end, the apparent rate of

regeneration using volume of stem as a measure of size would always be greater at the distal end even if the time for regeneration and the length of the primordium were the same as those of the proximal end. Such a difference in rate seems to me to be entirely due to morphological reasons and would not represent at all the inherent regenerative activity of the tissues themselves.

From the results (Table I, Fig. 2), however, it is clear that the difference in rate at various levels is a real one and that both the time for regeneration and the length of the regenerate vary in different regions of the stem. When R_1 is used as a measure of rate, the difference in distal and proximal levels of the stem is not so great as R_2

TABLE II

Relative rates of regeneration in *Tubularia*. Both hydranths are allowed to regenerate. D^r = relative rate of distal hydranth, P^r = relative rate of proximal hydranth. Rate = μ^3/hours·10^6.

Experiment	Length	No.	D^r	P^r	Thickness in μ		Vol. Stem
					D	P	
	mm.						$\mu^3 \cdot 10^7$
1	5	6	68.5	0	—	—	—
2	5	21	60.5	0	—	—	—
3	6	25	95.0	4.65	568	528	141
4	10	20	55.0	7.3	488	480	184
5	15	10	40.6	1.9	384	296	136
6	15	10	46.7	18.8	424	368	184
7	15	10	67.0	12.6	476	384	216
8	15	10	70.4	0	560	376	276
9	14	20	93.0	4.9	576	460	296
10	20	10	49.5	19.0	416	344	216
11	25	10	72.5	27.9	460	380	346
12	25	10	67.8	20.4	508	372	380
13	25	10	105.5	29.0	564	424	470

shows. The rate of regeneration of the most distal hydranths $D_1{}^i$ is 36.0 R. U. while that of the most proximal hydranth $P_6{}^i$ is 4.6 R. U., or about an 8-fold difference.

From Table I it will be noted that for pieces of the same length the volume of the stem varies at different levels because the stem is narrower at the base. It might be argued that the reduction in volume of the stem was responsible for the reduction in rate of regeneration. The volume of the most distal 5-mm. piece is $39.4 \cdot 10^7$ μ^3, while the most proximal 5-mm. piece has a volume of only $26.0 \cdot 10^7$ μ^3. This is a small variation in volume compared to the change in rate from 71.6 R. U. to 4.2 R. U.

The Effect of Length of Stem on Rate of Regeneration of the Ends

Figure 3 shows the effect of varying the volume of the stem but keeping the level of the cut constant; i.e. using the same region of the stem but with more stem attached to it. This is accomplished by a series of ligatures as in Fig. 1, *F, G, H.* Inspection of Fig. 3 tells us that the rate of regeneration at a particular level is increased by increasing the volume of the stem but that the increase is small. For example, increasing the volume from 20.8 to 155 units produces a change in rate from 22 to 26 regenerative units for the proximal hydranths. Further increase of volume to 312 units increases the rate to 34 regenerative units. The distal hydranth behaves similarly.

TABLE III

Comparison of relative and absolute rates of regeneration. D^r = relative rate in $\mu^3/\text{hr} \cdot 10^3$; D^a = absolute rate. Same for proximal. Dominance = $\frac{P^a - P^r}{P^a} \cdot 100$.

Experiment	No. Stems	Length	D^r	P^r	D^a	P^a	Dominance
		mm.					*per cent*
A	6 whole stems	5	45.5	0	—	36.8	100
B	14 distal halves	7.5	40.5	0	38.6	31.9	100
C	14 proximal halves	7.5	23.4	3.9	24.9	15.1	74
D	10 distal thirds	10	52.7	10.7	57.0	35.9	70
E	10 middle thirds	10	36.1	4.4	26.0	13.0	66
F	10 proximal thirds	10	19.1	0	27.0	8.6	100
G	10 distal halves	13	86.7	41.7	90.5	53.0	22
H	10 proximal halves	13	41.4	17.9	53.6	28.5	38
I	10 whole stems	15	67.0	12.6	63.0	26.8	54
J	10 whole stems	15	46.7	18.8	40.4	20.4	7
K	10 whole stems	20	49.5	19.0	51.2	28.9	34
L	10 whole stems	25	105.5	29.0	108.0	47.4	39
M	10 whole stems	25	72.5	27.9	66.8	34.0	20

Compare these small changes in rate with the increase obtained with change in level (Fig. 2). It is of interest here that the total increase in rate for the distal and proximal hydranth, when the 312 units of stem are used, is the same and amounts to about 12 units. This value may express the amount which the middle of the stem contributes to the ends in regeneration.

The Relative Rates of Regeneration of Pieces of Tubularia

Knowing the differences in inherent rates of regeneration and also the effect of volume on rate, we are in a position to study relative rates and the factors that determine these rates. The rate is termed "relative rate" whenever both ends of a piece of stem of *Tubularia*

are allowed to regenerate without a ligature. (See Fig. 1; *A*, *B*, *C*, *D*.) The superscript "*r*" is used. In this case two hydranths are regenerating and are doing so in relation to one another, since they are connected by the stem. Here competition and resultant dominance come in, especially with short stems.

Relative Rates of Whole Stems.—In Table II the relative rates of whole stems of different lengths have been recorded. The stems are prepared as in Fig. 1, *A*. The dominance of the distal regenerating end becomes greater as the stem is cut shorter, so that at 5–10 mm. proximal hydranths often do not appear. As the length of the stem is increased, the rate of regeneration of the proximal hydranth increases. Other factors, such as thickness and volume of stem, play a part.

Relative Rates of Regeneration in Distal and Proximal Halves.—When the stem of *Tubularia* is cut into halves, four hydranths may develop, which we designate as D_1, D_2, P_1 and P_2, as in Fig. 1, *B*. The rates for such stems of various lengths are given in Table III. To show the steepness of the gradient and also the phenomenon of dominance, the relative rates are plotted in Fig. 4. In long stems (26 mm.), there is very little difference in the relative rate of regeneration of the distal hydranth of the proximal half, D_2, and the proximal hydranth of the distal half, P_1. This is to be expected if there is no dominance, since these are adjacent levels of the stem (Fig. 1, *B*). The rapidly developing distal hydranth, D_1, in the distal half does not inhibit the proximal hydranth, P_1, any more than the slowly developing proximal hydranth, P_2, in the proximal half inhibits the distal hydranth, D_2. There is thus no apparent dominance exerted in the ordinary sense, i.e. as an inhibition exercised by a more rapidly regenerating region.

However, in shorter stems dominance appears, and the relative rates of regeneration of the distal hydranth of the proximal half, D_2, and the proximal hydranth of the distal half, P_1, are then quite different. In Fig. 4 the rate of $D_2 = 23.4$ R. U., $P_1 = 0$. In the distal half the distal hydranth D_1 is completely dominant over the proximal P_1. In the proximal half of the stem the distal hydranth, D_2, is only partially dominant over the proximal hydranth, P_2. The rate of $P_2 = 4.0$ R. U.

Finally, in Fig. 4, we have plotted the rates of regeneration of the six possible hydranths, D_1, D_2, D_3 and P_1, P_2, P_3 of Fig. 1, *C* for 10-mm. lengths of stems originally 30 mm. long. The graded differences in rate of regeneration in different regions of the stem are shown by the rates of the distal hydranths and also by the proximal hydranths in Table III, Experiments *D*, *E*, *F*. It will be noted, however, that at any level at which stem is cut the distal hydranth of that level regenerates much more rapidly than a proximal hydranth at the same

level. This means that the distal regenerating hydranths in each third of the stem are inhibiting the proximals in that same third. The situation can be summarized by saying that $D_1^r > D_2^r > D_3^r$ and $P_1^r > P_2^r > P_3^r$, but that $D_2^r > P_1^r$ and $D_3^r > P_2^r$, showing that both D_1 and D_2 are dominant. P_3^r is 0, showing the dominance of D_3.

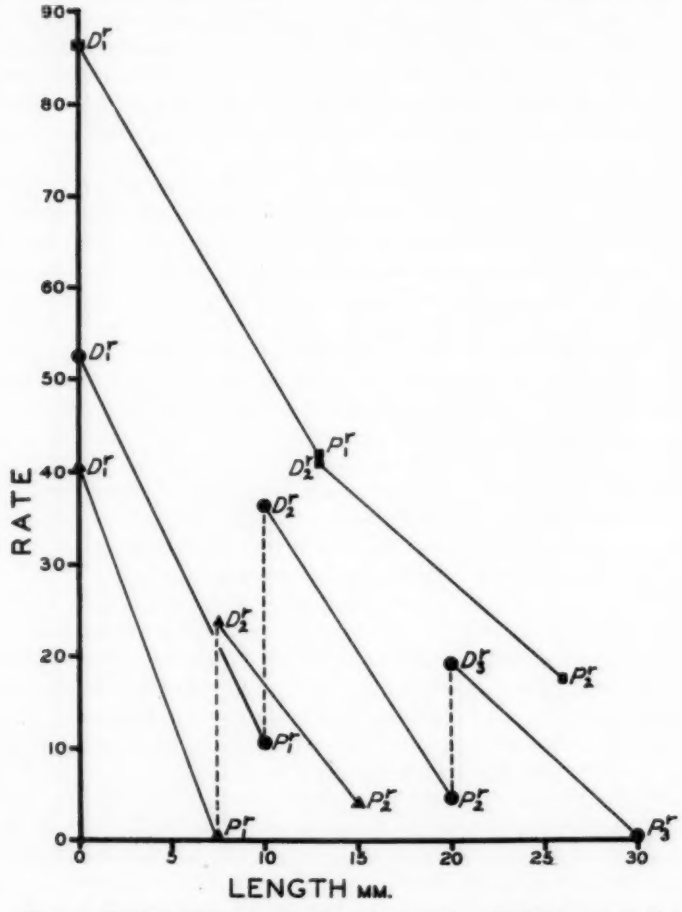

FIG. 4. Relative rates of regeneration of cut stems. Squares = stems 26 mm. long cut into halves; triangles = stems 15 mm. long cut into halves; circles = stems 30 mm. long cut into thirds. D_1^r, D_2^r, P_1^r, etc. = relative rates of respective hydranths. Rate = μ^3/hrs·10^5. The dotted line serves to connect adjacent regions of a cut stem.

The plot of rates in three sets of stems in Fig. 4 shows quite clearly that in each case the gradient is steeper in the upper (distal) levels of the stem. The slopes of the gradient in the upper levels of the three sets of stems are approximately parallel, which seems to indicate that the drop in rate per unit length of stem is about the same in different stems. It should be pointed out that the gradient here will be steeper than the gradient in inherent rates (Fig. 1), since dominance lowers the rate of proximal hydranths.

The Reciprocal Influence of Two Regenerating Regions

In stems of *Tubularia* from 10 to 30 mm. in length, a hydranth usually regenerates at both ends, and it is the purpose of the following experiments to show how the rate of regeneration changes when either the distal or proximal hydranth is prevented from regenerating by means of a ligature allowing the hydranth at the opposite end to regenerate free from dominance. These rates have been termed absolute rates, D^a, to distinguish them from relative rates, D^r. When only one end of a stem is allowed to regenerate, the rate is termed "absolute rate" in contrast to relative rate. This absolute rate of one end can be determined by simply tying off the opposite end of a stem, which prevents regeneration at this end. This rate tends to be the maximal rate of regeneration of a given region since the region has the entire length of the stem affecting its rate and is also independent of a second regenerating region (Fig. 1, *H*, D^{22}). It will also be seen that the absolute rate is simply the inherent rate plus the increase due to the addition of the stem.

The Influence of the Distal Regenerating Region on the Proximal End.—In general (Table III), the shorter the stem the greater the inhibiting effect of the distal regenerating region, and the following cases are arranged according to the length of the stem. In each experiment three lots of stems were used. The first lot regenerated without a ligature, giving relative rates D^r and P^r. The second lot regenerated with the distal end tied off, giving the absolute rate of the proximal end P^a, while in the third group the proximal end was ligatured, giving the absolute rate of the distal end, D^a.

Without exception (Table III), the absolute rate of any proximal hydranth is higher than its relative rate. Indeed, in some cases the relative rate may be 0, while the absolute rate is fairly high, 36.8 units (Experiment *A*). This method of treating the proximal end furnishes a measure of dominance, for if we know the absolute rate and the relative rate we can calculate the percentage reduction due to the distal end, i.e. the inhibition exercised by a dominant region. In

Table III, last column, this percentage has been calculated, and dominance varies from 100 per cent in short pieces to 7 per cent in longer pieces.

The Influence of the Proximal Regenerating Region on the Distal End.—In contrast to the proximal hydranth, the distal hydranth is affected little if at all by the ligature at the proximal end. This is true in the upper levels of the stem. Thus, in Table III, Experiment *B*, the relative rate of the distal hydranth, $D_1^r = 40.5$ R. U., while the absolute rate of the same hydranth, $D_1^a = 38.6$ R. U. and also, Experiment *L*, $D_1^r = 105.5$, $D_1^a = 108.0$. However, in lower levels of the stem, the absolute rate of the distal hydranth is usually greater than the relative rate. Experiment *F*, the relative rate of the distal hydranth of the

TABLE IV

The relation of the inherent rates of regeneration to the relative rates in pieces of *Tubularia* of varying length. Rate $= \frac{\mu^3}{t} \cdot 10^6$. D^i = inherent rate of distal hydranth; P^i = inherent rate of proximal hydranth; D^r = relative rate of distal hydranth; P^r = relative rate of proximal hydranth.

Length	Ligature D^i	Ligature P^i	No Ligature D^r	No Ligature P^r	Ratios D^i/P^i	Ratios D^r/P^r	Remarks
mm.							
25	54.6	22.0	72.5	27.9	2.46	2.60	Chiefly partition, no dominance.
20	38.2	13.9	49.0	19.0	2.74	2.60	Chiefly partition, no dominance.
14	81.5	22.6	93.0	4.9	3.61	19.0	Dominance incomplete.
5*	71.6	47.0	78.0	0	1.53	∞	Dominance complete.

* t_1 used in place of t_2.

proximal third of the stem $D_3^r = 19.1$, the absolute rate of the same hydranth, $D_3^a = 27.0$; Experiment *H*, $D_2^r = 41.4$, $D_2^a = 53.6$. This is extremely interesting, because it shows that the proximal hydranth does have an inhibiting influence over the distal hydranth in lower levels of the stem. In proximal regions of the stem then there is a reciprocal influence of the two regenerating ends on each other, each tending to inhibit the other.

The Competition between Two Regenerating Regions Having Different Rates

In the second section of this paper it has been shown that adding parts of the stem to either the proximal or distal end increases the rate of regeneration. Therefore the stem as a whole contributes materials to the regenerating ends, and the way in which this material is par-

titioned can be studied in stems of different lengths which provide different amounts of the material. The following experiments were designed to determine how the distal and proximal hydranths would divide or partition the effect of adding a definite volume of the stem to the system. For this purpose the inherent rates of regeneration of the proximal and distal hydranth were found by tying 3-mm. ligatures at the ends of the stems as in Fig. 1, *F* and *H*, D_1^i and P_1^i. In the same stems the opposite ends D_1^{22} and P_1^{22} give the rates of regeneration with 19 mm. of stem added to each end. Finally, as in Fig. 1, *A*, the two ends were allowed to regenerate in competition for the intervening 19 mm. of stem.

It is seen from Table IV that in long stems (20–25 mm. lengths) the addition of the middle of the stem to the distal and proximal ends

TABLE V

Increase in rate of regeneration of distal and proximal ends when the same volume of stem is added to each.

Total Length	Stem 3 mm. Inherent Rate		Volume Added $\mu^3 \cdot 10^7$	Stem 22 mm. Absolute Rate	Increase in Rate
mm.					
25	Distal	54.6	280	66.8	12.2
	Proximal	22.0	266	34.0	12.0
	Stem 3 mm. Inherent Rate			Stem 17 mm. Absolute Rate	
20	Distal	38.2	163	51.2	13.0
	Proximal	13.9	176	28.9	15.0

increases the rate of regeneration of these ends in direct proportion to their inherent rates. That is, the relative rates are directly proportional to the inherent rates $D^r/P^r = D^i/P^i$. Therefore there is simply a partition of materials without any dominance. This result would be expected if no factor other than available foodstuffs controlled the rates of regeneration.

However, in short stems (14 and 5 mm. lengths) there is no longer a partitioning of substances in proportion to the inherent rates of the distal and proximal hydranths, but rather a dominant effect of the distal end, so that it takes more than its share. $D^r/P^r > D^i/P^i$. As a matter of fact, in all non-ligatured 5-mm. pieces examined in this experiment and many others, no regeneration occurred at the proximal end at all, in spite of the fact that the proximal end had an inherent rate almost as great as the distal $D^i/P^i = 1.53$. This situation represents complete dominance in which all of the material goes to the distal hydranth.

A Comparison of the Effect of the Distal Half and the Proximal Half on the Inherent Rate of Regeneration of the Ends

It has been shown that the middle regions of the stem contribute materials for the regeneration of the two ends. The amount which the stem contributes can be measured by the increase in the rate of regeneration of the hydranths under the assumption that rate is proportional to amount of substances available. Thus, in Table V, the inherent rate of regeneration of the distal end is 54.6 R. U., and when we add 19 mm. of stem, the rate increases to 66.8 R. U. or an increment of 12.2 R. U. Similarly, P^i increases from 22.0 to 34.0 R. U., or an increase of 12.0 R. U. The increase is the same for both proximal and distal ends. (See 20-mm. stems also.) It is clear that the materials of the stem can be used by either the proximal or distal end.

Now if we ligature the stem in the middle, the effect of the materials in distal and proximal halves on the regeneration of the ends may be

TABLE VI

Comparison of the effect of distal and proximal halves of a stem on the rate of regeneration of the ends. D^i is the inherent rate of regeneration of the distal end. Under distal half is the rate of the distal end, with middle ligature. Similarly P^i is the inherent rate of the proximal end. Proximal half = rate of proximal end with middle ligature.

	D^i	Distal Half	Increase	P^i	Proximal Half	Increase
25 mm.....	54.6	64.6	10	22	26	4
14 mm.....	82.0	98.0	16	22.6	24.2	1.6

studied (Table VI). It is found in the two experiments available that the increase in rate is much greater in the distal as compared with the proximal half. This may be taken to mean that there are more materials available in the distal half than the proximal. The curves for the effect of volume on rate (Fig. 3) also indicate that the distal half is more effective in increasing rate than the proximal. As volume is added to the distal end, the rate goes up sharply and then falls off. However, as volume is added to the proximal end, the rate goes up slowly at first and then sharply. The evidence seems rather conclusive that the two regions differ in their effects on rate of regeneration.

Isolation of Distal and Proximal Regions by Means of a Middle Ligature and its Effect on the Relative Rate of Regeneration of the Ends

This method of studying dominance was used in the first experiments, and it is complicated by the fact that not only is isolation pro-

duced by the ligature but the volume of the stem is also reduced. The effect on the proximal hydranth is a dual one. Isolation increases the rate of regeneration, while reduction in volume decreases the rate, and the effect is a summation of the positive and negative action. Since, as we have shown, the reduction of the volume of the stem adjacent to the proximal hydranth decreases the rate to a small extent only, the chief effect is to remove the dominant region from the sphere of action, and the net result is an increase in the rate of the proximal hydranth (Table VII).

Length is an important factor, as in 25-mm. stems the change in rate with ligature is slight, while in 15-mm. stems it may be much greater, depending on the condition of the stems. This is in keeping with the fact that little dominance is exerted in long stems.

TABLE VII

Rate of regeneration of distal and proximal hydranths of *Tubularia* with and without a middle ligature. D^r and P^r = relative rate; D^l and P^l = rate under conditions of middle ligature.

Experiment	Length	No.	D^r	D^l	P^r	P^l
	mm.					
1	25	10	67.8	70.5	20.4	25.4
2	25	10	72.5	64.6	27.9	26.0
3	15	10	46.7	43.2	18.8	21.0
4	15	10	63.0	64.0	17.5	23.0
5	15	10	40.6	36.4	1.9	15.0
6	15	10	47.6	38.6	0	15.1
7	14	20	93.0	98.0	4.9	24.2

The Mechanism of the Dominance Exerted by the Distal Regenerating Hydranth over the Proximal End of the Cut Stem

In all previous experiments a ligature was used to block the dominance exerted by the distal regenerating end over the proximal end. With ligature of the stem the proximal end is allowed to regenerate independently of the distal and its rate of regeneration is greatly increased. This ligature, however, not only stops circulation between the distal and proximal end but also severs cellular connections. Thus it is not clear whether the factor responsible for dominance is something present in the circulation or something transmitted or transported through the cells. Therefore it is necessary to determine the effect on the proximal end of stopping circulation from the distal end but leaving cellular connections intact.

Use of a Loose Ligature for Blocking Circulation.—It is not easy to

shut off circulation between parts of the stem of *Tubularia* as the cells will rearrange themselves after compression of the stem so that the circulation breaks through once more. However, there are indications which can be seen from Table VIII where the effects of a loose ligature which was tied so as to just stop circulation is compared with a tight ligature cutting through the cœnosarc and breaking all cellular connections. The control with no ligature shows that we are dealing with stems in which the distal end is almost completely dominant over the proximal end: i.e. distal end, 38.7 R. U.; proximal end, 2.9 R. U. A tight ligature completely isolating the two halves sends the rate of regeneration of the proximal end up to 23.0 R. U. or an 8-fold increase. The distal end shows a slight reduction to 34.0 R. U. as it is cut off from the proximal half of the stem. Now with a loose ligature where cellular connections are still intact the proximal end shows an increased rate of 23.0 R. U. over controls in spite of the fact that at the end of the experiment circulation was reëstablished in a few cases through

TABLE VIII

Rate of regeneration of distal and proximal ends of *Tubularia* under conditions of ligature in the middle of the stem. Rate = L/t_2 where L is length of primordium in micra and t_2 is time in hours from cutting of stem to emergence of hydranth.

No. and Length of Stems		No Ligature		Tight Ligature		Loose Ligature	
		D	*P*	*D*	*P*	*D*	*P*
10	15 mm.	38.7	2.9	34.0	23.0	36.4	23.0

the ligature. This sort of experiment, while it appears conclusive, is not entirely satisfactory as it is difficult to control the tying of a ligature so as to cut off circulation without breaking cellular connection between the two halves.

Injection of Oil to Block Circulation.—The stem of *Tubularia* is about 0.5 mm. in diameter and it is relatively easy to insert a micropipette for injection. It is necessary merely to crack the rigid perisarc first with two pairs of sharp watchmaker's forceps after which a pipette can be inserted while observing under a binocular microscope. A small drop of paraffin oil (Nujol) is injected and after the pipette is withdrawn the rigid perisarc snaps back into place. The perisarc must not be removed because then regeneration will take place at the exposed surface. Controls for this type of experiment were stems in which the perisarc was ruptured and the pipette inserted without injection. Some of the experiments where oil was injected also served as controls since the drop was sometimes too small to block circulation.

Table IX records results. There are 25 stems in each sample and of the 25 controls only 2 proximal hydranths developed, making the rate 4.65 R. U. and thus showing that the distal end exerted rather complete dominance. When circulation is blocked, however, the 25 injected stems regenerate 12 proximal hydranths, bringing the average rate to 22.8 R. U., or a 5-fold increase in rate. The distal hydranth in the injected group shows a small decrease in rate, as might be expected from previous results on the use of ligatures. A ligature in a short stem increases the rate of the inhibited proximal end but decreases the rate of the dominant distal end by shortening the stem.

Only 12 out of 25 possible hydranths appear at the proximal end of injected stems and it is interesting to examine the 13 stems which did not form a hydranth proximally. Of these 13 negative cases 9 showed that the oil drop had moved from its original position at the middle of

TABLE IX

Injection of an oil drop into the gastrovascular cavity of *Tubularia*. Stems 6 mm. long. Twenty-five stems used in each sample. R = rate of regeneration $= \pi r^3 L/t_2$ where r = radius of stem in micra; L = length of primordium in micra and t_2 = time in hours required for emergence of the newly regenerated hydranth.

	Oil Injected		No Injection	
	Distal	Proximal	Distal	Proximal
L	1384	528	1536	104
r	282	264	284	280
t_2	40.0	50.5	40.9	55.0
R	86.1	22.8	95.0	4.65

the stem into the distal end. In the 12 stems which form a proximal hydranth all but 2 showed the oil in the original position. It is clear that because the size of the oil drop varies the smaller drops do not completely block circulation and as a result they are carried by the circulation to the distal end. When the drop is larger it is held firmly in place by the endodermal lining of the gastrovascular cavity and in these cases proximal hydranths appear associated with a complete block to circulation.

In other experiments where dominance is not so complete there is a quantitative effect on rate of regeneration of the proximal end, with injection of oil into the gastrovascular cavity. It must be remembered that this effect is not so great as would be expected since not all of the oil drops are large enough to block circulation. An example is shown in Table X, where the proximal ends of control stems regenerate at a

TABLE X

The effect of blocking circulation in *Tubularia* with oil drops. Oil injected into distal region. Controls consist in injury similar to that of injection. Twenty-one stems, 10–11 mm. in length used for each sample. $R = \pi r^2 L/t$.

	Oil Injected		Injury Control	
	Distal	Proximal	Distal	Proximal
L	1200	1032	1352	612
r	254	236	252	228
t_1	35.4	47.0	37.3	50.0
R	67.0	38.4	72.0	20.0

rate of 20.0 R. U., but upon injection of oil increase to 38.4 R. U. The rate of the distal hydranth is reduced from 72.0 R. U. in untreated

TABLE XI

Effect of blocking circulation by means of oil in *Tubularia*. Stems 8–10 mm. long isolated from long thick stems. Twenty-eight stems in each sample. R = rate using t_1; l = length of primordium in micra; r = radius of stem in micra; t_1 = time in hours from cutting to formation of primordium. Rate = μ^3/hrs.·10^6.

	Oil		Control	
	Distal	Proximal	Distal	Proximal
L	1388	884	1320	852
r	286	276	286	268
t_1 hours	37.8	46.9	37.3	46.0
R	94.0	45.0	91.0	41.7

stems to 67.0 in injected stems. This decrease is in part caused by injection of the drop into the distal region of the stem which isolates a small portion. The average length of stem from the oil to the distal end was 2.5 mm. at the termination of the experiment.

Finally, in stems where there is very little dominance there is little effect of injection of oil in the middle of the stem. This result was obtained from some 8–10 mm. pieces cut from long, thick stems. Table XI shows that neither the proximal nor distal hydranths are affected to any extent by the injection of oil. This result is to be expected from the section dealing with the use of a middle ligature to isolate the two ends. In stems where the distal hydranth exerts little dominance it was found that little effect was produced on the rate of regeneration of the proximal hydranth by a ligature.

The experiments on injection of oil into the gastrovascular cavity

of *Tubularia* shows that the oil isolates the proximal end of the stem from the distal end, producing about the same effect as a ligature. In the former case the circulation is blocked while in the latter both circulation and cellular transmission are blocked. It becomes important then to see just what the oil drop does in the gastrovascular cavity. It has already been pointed out that when the drop is small it has little effect in blocking dominance. Also, when there is little dominance, little or no effect of injection of oil is found. Therefore it is safe to say that there are no toxic chemical or physical effects of the oil on the cells which come in contact with the oil.

Examination of the region into which the oil is injected shows it to be firmly held in place by the endoderm which it partially displaces. When it is not so held in place it moves during the course of hours to the distal end of the stem. Although the endoderm is displaced and perhaps the cellular connections in this layer are broken, the ectoderm remains intact and the cells can be seen to be continuous over the surface of the oil drop. As we were not satisfied with this observation, the conductivity over the bridge of ectoderm was tested by using an electrical stimulus. In some previous unpublished work on electrical stimulation in *Tubularia* it was found that upon applying a stimulus at the proximal end the tentacles of the distal hydranth would respond. Three stems in which dominance was blocked by injection of oil were treated in this manner and in each case the tentacles of the distal hydranth responded to an electrical stimulus applied at the proximal end. From these observations there can be little doubt that the ectodermal connections over the oil drop are morphologically and physiologically intact and that dominance is not transmitted over this layer.

Discussion

Child (1907) pointed out that in *Tubularia mesembryanthemum* both the length of the cut stem and the level at which the stem was cut were factors determining the time for regeneration and size of the primordium. Driesch (1899) before this had measured the primordium of halves of stems and found that the oral half (distal half) formed longer primordia than the aboral (proximal half). Driesch also showed that the hydranths emerged faster in the oral (distal) half as compared with the aboral (proximal) half. Thus the regional differences in regenerative capacity in the stem of *Tubularia* are by no means new. The new treatment of the facts by combining two variables, size and time, into a rate has not been suggested hitherto. By utilizing both variables it is possible to express the rate of change within the stem at any level and so compare rates under various conditions. It is hoped that the

rate as defined in this paper is a measure of the chemical changes involved in the differentiation of the hydranth from the stem after cutting. It is not sufficient to express these changes in terms of time only since two hydranths of different sizes may regenerate in the same time and certainly the larger hydranth must have utilized more material than the smaller. Therefore the rate of chemical change must have been higher in the region which formed the larger hydranth. Similarly two hydranths of the same size may require different times for regeneration.

A second difference between these experiments and those of early investigators is the use of a ligature to isolate regions in order to test their rate of regeneration. Driesch (1899) and Child (1907) cut stems into halves and thirds and allowed both ends to regenerate. The size and time for regeneration of distal (oral) hydranths is modified by the presence of a second region of regeneration. Both investigators showed that the size and time factors varied with the length of stem cut. In my experiments by the use of the ligature the second regenerating region is eliminated and the size kept constant. Thus the "inherent" rate is measured. It is proposed that the "inherent" rate of regeneration be used as a base so that the effect of variables such as length of cut stem and the rate of regeneration of a second hydranth can be studied by means of appropriate ligatures.

Since the stem of *Tubularia* shows a gradient of "inherent" rates there must be graded differences in the concentration of some substance or substances, which account for these different rates. Further, because the rates are higher in the younger (distal) regions of the stem it is reasonable to assume that the substances are of the nature of a synthetic factor which is able to convert available materials into a hydranth.

We will let this inherent synthetic factor be represented by E and assume that the concentration of E is proportional to the inherent rate of regeneration as measured by isolation of parts of the stem. E is then present in highest concentration in the young cells at the distal end and in lowest concentration at the proximal end. Then Fig. 2, giving rates of regeneration, may be taken to indicate the relative concentration of E at various regions of the stem since only internal factors are responsible for these differing rates at various levels of the stem.

But E is not the only factor affecting rate. In Fig. 3 it was shown that increase in length of stem adjacent to the regenerating region will also increase rate of regeneration. It is evident that something from the middle of the stem travels to the ends and causes an increase in rate. Let us call this factor or substance S. S is transported through

the gastrovascular cavity in the circulation which is easily observed in *Tubularia*. A cross-section of the stem shows four channels in the endoderm and in the intact stem particles can be seen travelling up one side of the stem and back down the other so that a fairly rapid circulation exists. Timing the flowing particles gave a velocity of 6 mm./minute. This means that in a short stem 6 mm. long a complete circulation of the contents of the coelenteron should take place in 2 minutes. If one end was using up materials in rapid regeneration it is conceivable that the concentration of substance, S, in the circulation would be lowered considerably so that at the opposite end substances might pass into the gastrovascular cavity from the cells and so inhibit regeneration by removal of available materials.

The effect of S on rate of regeneration is difficult to measure but we may take Fig. 3, which shows increase of rate with increased volume of stem as a provisional measure of S. Obviously, however, this does not give us the effect of S in very low concentrations. That S is a very important factor is seen by comparing the inherent rate of the proximal end with the relative rate (Table IV, 14-mm. and 5-mm. stems). In these cases something is actually removed from the proximal region so that although the stem is much larger the rate of regeneration is lower when the distal hydranth is regenerating.

The situation may be summarized as follows. Regeneration is essentially the transformation of stem into hydranth and this requires chemical changes: $S \overset{E}{\underset{\downarrow}{\rightleftarrows}} H$. Let us assume E to be a catalyst in the cell which transforms S into H, H being the substances necessary for hydranth differentiation. The reaction is reversible, as Child (1923) has shown that hydranths may dedifferentiate into stem. I have also observed a hydranth partially differentiate from cœnosarc and then return to cœnosarc. E, as we have pointed out, is present in the cells and is in highest concentration at the distal end. S is in the cells and also the gastrovascular cavity and is present in greatest amount in the longest stems.

In long stems, where S is high (Table IV, 25-mm. and 20-mm. stems) it appears that S is partitioned to the distal and proximal hydranths according to their inherent rates of regeneration, as represented by E. One way of expressing it is that, with increase in S while Ep (concentration of E at the proximal end) and Ed (concentration of E at the distal end) remain constant, H_p (concentration of H at proximal end) and H_d (concentration of H at distal end) increase proportionally. This result is the expected one.

The difficulty comes when we consider short stems, where E_p (concentration of E at the proximal end as measured by the proximal inherent rate) is close to E_d (concentration of E at the distal end as measured by the distal inherent rate) yet apparently S is used by the distal hydranth and not by the proximal hydranth. All that can be assumed is that the factor E_d converts S into H as fast as it appears in the cœlenteron, so that the concentration of S is always below the minimal value necessary for regeneration at the opposite end. Since $E_d > E_p$ it can synthesize H at a lower concentration of S. By writing the reaction as a reversible one, we may even suggest that $H \rightarrow S$ at the proximal end in the case of short stems. This whole thought depends on the assumption that the difference between E_d and E_p in short stems, although small, is great enough to lower the concentration of S below a minimal value, S_m for the proximal hydranth. As S increases in amount (in longer stems) the concentration in the cœlenteron rises above the minimal value S_m for the proximal hydranth and regeneration starts at the proximal end though at a low rate at first. S thus becomes a limiting factor for regeneration in low concentrations.

It will be seen that the formal explanation given above is readily applied to other results, such as those from middle ligatures and the results showing the difference between relative and absolute rates of regeneration.

From the comparison of the absolute rate of the distal hydranth D^a and the relative rate of the same hydranths D^r in Table III, it is apparent that there is a maximal rate of regeneration for the distal hydranth, or a maximal concentration of S above which synthesis of H is not increased perceptibly. This is not unlikely.

The above discussion throws the entire effect of dominance on the transport of available substances (S) for regeneration. In *Tubularia* the transport system is extremely simple, and it seems an ideal system for experimentation. If in short stems the circulation of S is blocked (as by an oil drop) both the distal and proximal ends should regenerate independently of each other and no dominance will be exerted by the distal end. The experiments in this paper indicate that such is the case and thus show that S is a factor which circulates in the fluid of the gastrovascular cavity.

Summary

1. The rate of regeneration of *Tubularia* has been measured by the formula $R = \pi r^2 L/t$ where r = radius of cross-section, L = length of regenerate, t = time in hours for formation of primordium or the time in hours for emergence of the fully formed hydranth.

2. The rate of regeneration of isolated parts of the stem decreases

rapidly from the distal to more proximal regions. An increase in the length of stem adjacent to a regenerating end increases the rate of its regeneration.

3. When two regenerating regions are competing they partition the effect of adding the middle region of the stem if the stem is long. If the stem is short the distal end becomes dominant and inhibits the proximal end.

4. A method has been devised for measuring dominance by expressing it as the percentage reduction in rate of regeneration due to the presence of the distal hydranth.

5. The circulation within the stem of *Tubularia* has been blocked by means of injection of a drop of oil with the result that the dominance (inhibition) exercised by the distal regenerating end over the proximal end is blocked.

6. A generalized mechanism based on a synthetic factor E in the cells and a circulatory factor S is suggested as a formal explanation of the phenomenon of dominance.

LITERATURE CITED

CHILD, C. M., 1907. An analysis of form regulation in Tubularia. IV. Regional and polar differences in the time of hydranth formation as a special case of regulation in a complex system. *Arch. Entw.-mech.*, **24**: 1.

CHILD, C. M., 1923. The axial gradients in Hydrozoa. *Biol. Bull.*, **45**: 181.

CHILD, C. M., 1929. Physiological dominance and physiological isolation in development and reconstitution. *Arch. Entw.-mech.*, **117**: 21.

DRIESCH, HANS, 1899. Studien über das Regulations-vermögen der Organismen. *Arch. Entw.-mech.*, **9**: 103.

MORGAN, T. H., 1902. Further experiments on the regeneration of Tubularia. *Arch. Entw.-mech.*, **13**: 528.

PEEBLES, F., 1931. Some growth-regulating factors in Tubularia. *Physiol. Zoöl.*, **4**: 1.

CYTOLOGICAL INVESTIGATIONS OF COLPODA CUCULLUS

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Inasmuch as the various species of *Colpoda* have interested many previous investigators, especially with regard to factors of encystment and excystment, it was thought worth while to make a careful study of the best known species, *Colpoda cucullus* Muller. So many interesting and hitherto unreported phenomena have been observed that it was thought advisable to present only the cytological results in this first report. Subsequent reports will deal with the many and interesting observations on other phases of the problem which are at hand and these will be supplemented by further and more complete data.

The life histories of various species of *Colpoda* have been investigated from time to time starting with the work of Stein in 1854. He reported the encystment of *Colpoda cucullus*, the subsequent division into two, four, eight and even sixteen smaller individuals and their ultimate escape from the ruptured cyst. Rhumbler (1888) made an extended study of the process of encystment and division in *Colpoda cucullus* and *C. steini* and distinguished between the division cysts, "Theilungscyste," and those cysts within which division does not occur, the permanent cysts, "Dauercyste." He describes in some detail the appearance and activity of freshly encysted and dividing forms, noting that the cilia are retained throughout the division process but are lost during their stay in the permanent cysts. He observed quite accurately the gradual loss of the food inclusions, during permanent cyst formation, although his interpretation of the method of this loss is open to serious question.

Wenyon (1926) has given a rather diagrammatic account of the division of *Colpoda steini*, agreeing with the accounts of Stein and Rhumbler but adding some details of the nuclei. Wenyon's observations were made on fixed and stained material while those of Stein and Rhumbler were mostly obtained from the living material.

Very recently Penn (1937) has reported the occurrence of binary and quadruple division without encystment in a strain of *Colpoda cucullus*. Encystment before division occurred in his race only when the cultures were old or when the cultures became crowded. He was

able to induce the formation of cyst walls by placing "healthy individuals" in infusions containing gelatinous masses of bacteria. In this report are given descriptions of the nuclear phenomena which take place during the divisional process, but details are lacking.

There has been no cytological report of the nuclei of the "permanent" or resistant cysts published to date, as far as we are aware, and the reports of the nuclei during division have all been fragmentary. It is not difficult to see why resistant cysts have eluded observation in the past when one considers the fact that most nuclear investigation has been done after the employment of the various hæmatoxylin stains. As pointed out by Goodey (1913) the cyst walls ("ectocyst and endocyst") stain intensely with both Heidenhain's and Delafield's hæmatoxylins. We have found it impossible to study the contents of the resistant cysts after treatment with these standard stains and no doubt previous workers have experienced like difficulty. It is with the aid of the nucleal reaction developed by Feulgen and Rossenbeck (1924) that the details of the nuclear complex of resistant cysts have been rendered observable.

Hæmatoxylin stains do not react on the division cyst wall as they do on the resistant cyst walls, mainly because of the comparative delicacy of the former. It is possible to obtain a rough idea of what happens to the nuclei during division by employing these stains. But because of the densely packed food inclusions in the cytoplasm during this period fine details are obscured. After the Feulgen reaction, however, the history of the nuclear components may be readily followed. It has been found that this history is a rather surprising one and one that may shed considerable light on the rôle of the macronuclear chromatin in ciliate metabolism. Therefore, because we have observed with considerable exactitude the nuclear phenomena both during division and during the stay within the resistant cyst, and because we feel that these observations will contribute to our understanding of related phenomena among ciliates in general, we offer the following cytological report as the first one of a comprehensive nature on this common organism, *Colpoda cucullus*.

Material and Methods

Colpoda cucullus is a very common form and may be collected in a great variety of places. Our original material was obtained from dry hay taken from the banks of a brackish stream near Stuart, Florida. The hay was placed in spring water from which enormous numbers of the ciliates were later collected and transferred to small dishes. From these dishes a number of motile specimens were selected with a micropipette and placed in individual isolation culture dishes in a drop of

twenty-four-hour-old diluted hay tea. After a number of encystments and segregations had taken place one motile ciliate was selected and all of the others discarded. All glassware was then scrupulously cleaned and boiled for a long period of time to remove any danger of contamination. This obvious precaution was to insure the presence of only one species with which to work. All of our material, therefore, has descended from a single organism.

The standard culture medium used in this work, while not the only one successfully used, has given consistent results and the isolation lines grown in it have exhibited a surprising vitality. It consists of nothing more than ordinary hay tea, used after twenty-four hours at room temperatures. Into this medium single motile ciliates were placed and invariably at the end of twenty-four hours three to four divisions had occurred. All that was necessary to obtain resistant cysts was to allow multiplication to proceed for forty-eight hours or longer without adding fresh medium. Under those conditions the division rate decreased until finally all of the ciliates secreted the heavy wall characteristic of the resistance phase, accompanied by the other phenomena described below. Because of the high fission rate, the hardiness of the species and the predictable response to certain environmental conditions there was always an abundance of material in every phase of the life history under investigation.

For the preparation of permanent slides for cytological study we employed special "recovery dishes"[1] for the collection of material designed by one of us (C. L. C.). Small circular flat-bottomed Pyrex dishes with straight sides, measuring 23 mm. × 12 mm. (inside measurements), were prepared. These dishes will accommodate ⅞ inch circular cover glasses, allowing them to rest on the bottom with very little free space about the edges. Into one of the dishes was placed a cover glass, in some cases thinly coated with fresh egg albumin. The dish was then filled with culture medium and inoculated with a single motile *Colpoda*. By frequent microscopic examinations the stages desired could be accurately noted, the cover glass taken out and placed in the fixing fluid. In this way we were able to recover hundreds of the different stages on each cover glass. Another advantage this method has is that the organisms do not tend to pile up but each adheres to the glass more or less separately. There is a minimum of debris which makes for clarity of the final preparations. The success of these "recovery" dishes caused us to give up entirely our earlier methods of concentrating by centrifuging and of selecting individuals under the dissecting microscope.

[1] These dishes, known as the Claff Recovery Dishes, are being put on the market by Clay-Adams Company, Inc., New York City.

As mentioned in the beginning, our clear preparations resulted from the use of the Feulgen nucleal reaction. We made many preparations with the various hæmatoxylins and carmines, and while these preparations were entirely satisfactory for the motile forms and proved very useful, they were mediocre for divisional phases and entirely useless for resistant cysts. We found that certain modifications of the standard Feulgen procedure were advantageous. After much experimentation it was found that the following times gave excellent results and we highly recommend them for future work on *Colpoda cucullus:* acid hydrolysis—15 minutes at 60° C.; fuchsin sulphurous solution—4 to 5 hours; sodium bisulphite-hydrochloric acid wash—15 minutes (at least three changes). It was found that the increased time of washing had a decided tendency to clear the cytoplasm of any trace of free fuchsin and rendered the preparations beautifully clear.

Many types of fixing fluids were used but it was found that wherever cyst walls were present the more penetrating varieties gave, as would be expected, the best results. Therefore most of our material was fixed in Schaudinn's with 5 per cent acetic, corrosive sublimate in 95 per cent alcohol with 5 per cent acetic acid added, or the Gilson-Carnoy mixture. The latter fixing fluid gave the best results on the resistant cysts.

Our Feulgen prepared material was very often counterstained with either fast green in 95 per cent alcohol, methylene blue in 70 per cent alcohol, or the acid component of the Borrel mixture (Calkins, 1930). The last-named stain was modified as to balance from the original, containing ⅓ indigo carmine to ⅔ picric acid. Counter stains reacted well in the cytoplasm of trophic and divisional forms, penetrating the thin cyst membrane of the latter with ease. The resistant cyst membranes appear to be entirely impermeable to fast green, methylene blue and the indigo carmine component of the Borrel mixture. The picric acid of the Borrel stain penetrates very readily, however, staining the cytoplasm an intense yellow. After this counterstain striking preparations are obtained if the material has been taken from a culture in which trophic forms, divisional cysts and newly formed resistant cysts are present. The cytoplasm of the trophic forms and the divisional cysts is a brilliant green while the cytoplasm of the resistant cysts is yellow.

It should be mentioned that numerous preparations employing the silver nitrate method of Klein were made on the trophic forms as an aid to our positive determination of the species at the beginning of the investigation. The extreme variability of the size and form of these ciliates is so great that specific identification becomes difficult without

careful study of the ciliary pattern. The question of variation of the ciliary pattern we hope to present in another report.

It has been found possible to check very accurately the times when the various nuclear changes occur during each phase by starting with resistant cysts, inducing them to excyst and fixing material at frequent, timed intervals. In carrying out these timed observations a large number of "recovery dishes" were prepared at one time and their contained organisms fixed in order, first noting the condition of each in the living state. Due to this procedure we have a complete series of slides demonstrating the nuclear activity from excystment, through several reproductive divisions and through a second resistant encystment. As a result of these observations we are certain that the sequence of events to be described is the normal one and occurs in a regular fashion in this ciliate. All the cytological details observed from the timed material have been repeatedly checked from mass cultures.

The Life Cycle—General

Conjugation has never been observed by us in our strain of *Colpoda cucullus* and as far as we have been able to determine it has never been reported in the literature. Enriques (1908) mentions conjugation as having been noted by him in *Colpoda steini* but not in *C. cucullus*. Therefore we will use the term "life cycle" to denote the sequence of events taking place from one resistant cyst stage back to another.

Colpoda cucullus appears to possess the ability to accommodate itself to a wide variety of environmental conditions. The great factor in this extreme accommodation seems to be its ability to secrete, in a minimum of time, protective or semiprotective cyst membranes. Under normal conditions all reorganizational phases accompanying reproduction and resistant encystment are carried out when the organism is enclosed in some type of membrane or membranes. Feeding, only occurring in the trophic stage, is accomplished in a very efficient manner, for within a few minutes after resistant excystment the cytoplasm will be found to be literally packed with spherical food vacuoles.

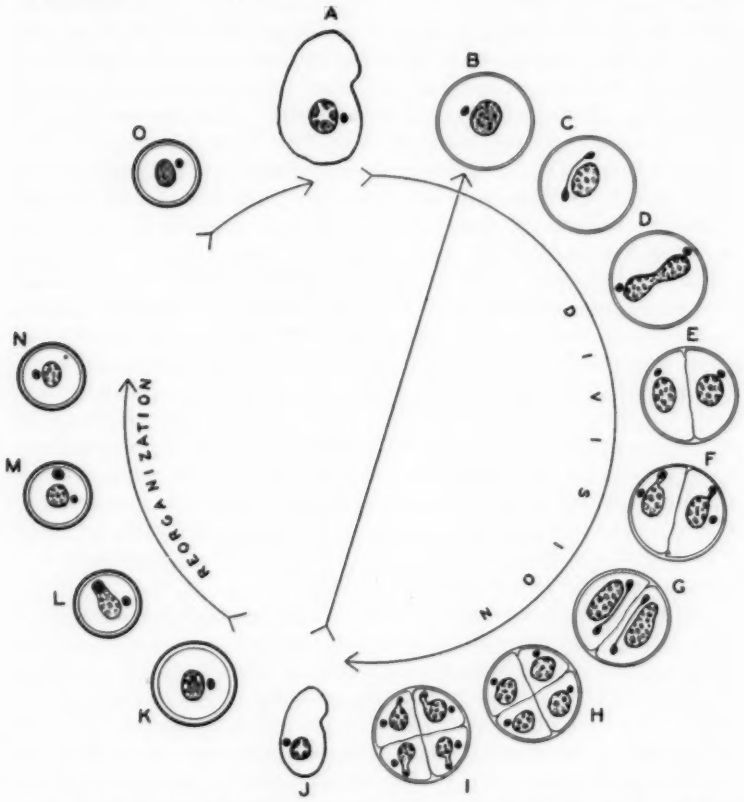
When placed in fresh culture medium the resistant cyst undergoes certain changes very rapidly. These changes lead to a rupturing of the heavy outer cyst wall (ectocyst of Goodey, 1913) and finally to liberating the swimming ciliate from the thin inner wall (endocyst of Goodey). One of us (C.L.C.) has been investigating the mechanism of excystment under normal and experimental conditions and will report these findings at an early date.

The newly excysted ciliate is devoid of food vacuoles but very rapidly engulfs great quantities of bacteria and bacterial debris. This

food is invariably formed into spherical compact masses and distributed at random throughout the cytoplasm. One average sized ciliate (70 μ) may have as many as two hundred such food vacuoles (Plate I, Figs. 1 and 2). Growth ensues, the ultimate size appearing to be dependent on the excellence of the cultural conditions but independent of the ability to reproduce. Within a few hours after excystment the trophic form begins to round up, without, however, losing the motility of the cilia. The cytostome becomes indistinct and a thin membrane is secreted outside the still moving cilia. This is the "Theilungscyste" of Rhumbler (1888) and undoubtedly corresponds to the "pseudocyst" of *Tillina canalifera* recently described by Turner (1937). In the vast majority of cases quadruple division occurs within this cyst resulting in the liberation of four small daughter ciliates. The cilia may be continually observed throughout the process. Occasionally binary division occurs resulting in the liberation of but two daughter ciliates in which case they are proportionately larger in size. We have never observed divisions resulting in more than four daughters although Stein (1854) has reported as many as sixteen daughter ciliates escaping from a division cyst. The report of Penn (1937), in which he maintains the normal condition in *Colpoda cucullus* to be quadruple division without encystment, was not confirmed in our study. Binary and quadruple division without encystments were encountered only occasionally among the great number of encysted forms irrespective of the bacterial condition of the medium. It is possible that lack of encystment is a peculiarity of the strain studied by Penn.

If no additions are made to the culture medium for a period of forty-eight hours the rate of division cyst formation decreases and stops and the ciliates become very small and less active. They round up very quickly and secrete a heavy, wrinkled cyst wall about themselves. This results in the resistant cyst which is able to withstand desiccation. The formation of resistant cysts may be induced by simply drawing off a part of the medium and replacing it with old medium from a culture in which resistant cyst formation has taken place. In the literature are numerous accounts of the necessity for drying before resistant cyst formation occurs (Barker and Taylor, 1931; Bodine, 1923; etc.). We have found that resistant cysts form readily even in an abundance of fluid, and may be kept for long periods without recourse to evaporation. As pointed out by Penn (1937), if conditions inducing resistant cyst formation occur rapidly enough (evaporation, according to Penn), some of the division cysts become resistant cysts by the simple expedient of forming a heavy wall about the outside of the division cyst wall. Thus resistant cysts may contain one, two

or occasionally four cells, all motor organelles dedifferentiating until nothing can be seen but the nuclear apparatus and the granular cytoplasm.



TEXT FIG. 1. Diagrammatic representation of the "life cycle" of *Colpoda cucullus* illustrating the sequence of events during reproduction and resistant cyst formation. *A–J*, normal reproductive activity repeated (*J* to *B*) under favorable cultural conditions. *K–O*, resistant cyst with space between *N* and *O* representing the lapse of an indefinite amount of time while the arrow from *K* to *N* represents a short space of time during which macronuclear reorganization and chromatin elimination takes place. Arrow from *O* to *A* represents the return of favorable conditions for excystment.

A young resistant cyst possesses many food inclusions but these are rapidly absorbed, leaving characteristic refringent inclusions in their stead. Within a few hours even the refringent bodies have disappeared

and the cytoplasm becomes compact and evenly granular, containing the nuclear apparatus. The compactness of the cytoplasm is brought about by the actual shrinkage in the size of the organism even after the cyst wall has been laid down. Resistant cysts are always very much smaller than all but the smallest of the trophic forms or division cysts.

The above account agrees essentially with the previously published descriptions of the "cycle" of *Colpoda cucullus* and will serve as a basis for the details of the nuclear activity described below.

Text figure 1 illustrates the normal course of events during the "life cycle" of *Colpoda cucullus* which, with the accompanying legend, will serve to clarify the above description.

The Nuclei of Colpoda Cucullus

Enriques (1908) gives as a specific characteristic of *Colpoda cucullus* the possession of a macronucleus with a lobed karyosome "cariosoma lobato." He was able to observe this after staining with carmine. From his figures it may be assumed that, except for the karyosome, the macronucleus is devoid of stainable material (chromatin). Wenyon (1926) figures the macronucleus of *Colpoda cucullus* with irregular karyosome-like bodies toward the center. He does not state what stain was employed but it would be safe to assume the use of a hæmatoxylin. That both of these observations were due to the type of stain used is demonstrated by a comparative study of organisms stained in borax carmine, Heidenhain's hæmatoxylin and after the Feulgen reaction. In the first two cases the stainable material seems to be concentrated toward the center of the macronucleus surrounded by a faintly stained, irregular periphery. But after the Feulgen reaction the picture is reversed. The chromatin is seen as irregular plaques surrounding and extending into the colorless nucleoplasm (Plate I, Fig. 1). After any of the counter stains used with the Feulgen reaction the nucleoplasm is sharply contrasted to the chromatin and is seen to have the form described as the karyosome by previous workers. Penn (1937) illustrates this general condition in his figures from Feulgen prepared material. We wish to emphasize the chromatin configuration of the macronucleus of the trophic form, therefore, as being in the form of irregular plaques around the periphery and surrounding the non-chromatin nucleoplasm of the center, because of the prevalent use of this character in classification (see the description of Kahl, 1931).

A single micronucleus is always present in the trophic stage. It lies near the macronucleus but may be on any side of it. The chromatin is quite compact and in fixed preparations is usually seen to have

shrunken away from the nuclear membrane. The micronucleus is quite commonly elongated, flattened on one surface, and slightly pointed at the ends. We have never found more than the single micronucleus in the trophic forms. Penn (1937) mentions the occasional occurrence of two or four micronuclei in his strain. We believe that it is possible that he interpreted the balls of extrusion chromatin from the macronucleus as micronuclei inasmuch as he completely overlooked these interesting bodies (see our Plate I, Fig. 23).

In our material the micronucleus behaves in an orthodox fashion during the divisional activities. After the division cyst membrane is laid down it enlarges and assumes a spherical shape. The chromatin becomes slightly less compact and is seen to be finely granular (Plate I, Fig. 3). It then becomes distinctly striated and the whole nucleus elongates and enlarges. The striations within the chromatin become more marked and finally irregular threads may be observed all oriented with the long axis of the nucleus (Plate I, Fig. 4). Contraction of the chromatin threads results in the formation of the metaphase plate (Plate I, Figs. 5, 6, 9). There appear to be a great many chromosomes and we were never able to make even an approximate count, as a glance at the figures will reveal. The anaphase is formed by a separation of the chromosomes of the metaphase plate into two groups; the nature of this separation has not been determined. The two daughter chromosome groups move to opposite ends of the fully formed spindle leaving between them definite clear fibers which remain into the elongated telophase (Plate I, Figs. 7 and 8). The chromatin becomes compact again in the daughter telophase groups and these move farther apart (Plate I, Figs. 10 and 19), retaining for some time the connecting strand formed from the nuclear membrane. Finally this strand breaks and the two micronuclei round up and take their positions at opposite ends of the now elongated macronucleus (Plate I, Figs. 11 and 12). This sequence is repeated prior to every cell division without any appreciable variation.

It is within the chromatin of the macronucleus that events occur that offer an interesting problem in ciliate cytology. Before the division cyst membrane is laid down the chromatin loses its plaque-like configuration and becomes flocculent, being roughly dispersed throughout the nucleoplasm. It is still slightly granular but the granules are exceedingly minute (Plate I, Fig. 2). By the time the division cyst membrane is formed the chromatin of the macronucleus has begun to take on a definite configuration (Plate I, Fig. 3), that of granular aggregates suspended in the clear nucleoplasm (Plate I, Figs. 9, 10 and 11). This configuration is retained until the daughter organisms are

ready to emerge from the cyst, in most cases through the two divisions. The chromatin aggregates stain uniformly and rather intensely but because of their scattered condition the macronucleus as a whole appears as a loosely knit body, lying among the numerous food inclusions. By the time the micronucleus has reached its telophase stage the macronucleus has begun to elongate. This elongation marks the future division plane of the cell, being always at right angles to it. Further elongation stretches the macronuclear membrane until a typical constriction appears separating the chromatin into two daughter halves (Plate I, Fig. 12). The two daughter halves of the macronucleus quickly separate and round up and the fission plane forms, dividing the cell into two equal daughter cells, each containing a single micronucleus and macronucleus and numerous food inclusions (Plate I, Fig. 13).

Shortly after cell division there becomes differentiated simultaneously within each daughter macronucleus an irregular, granular mass of chromatin. This mass stains much more intensely than the general macronuclear aggregates and is first seen lying close to the nuclear membrane. It is rapidly separated from the chromatin aggregates and is pushed out from the surface of the macronucleus being surrounded by a pocket formed from the macronuclear membrane. This activity is nearly or exactly synchronous in each daughter macronucleus (Plate I, Fig. 14). This deeply staining chromatin mass is the "extrusion chromatin" or "residual chromatin" and is ultimately broken away from the macronucleus and cast into the cytoplasm (Plate I, Figs. 15 and 17). Within the cytoplasm it becomes compacted into an intensely staining ball which rapidly diminishes in size until it disappears from view. It is usual that complete absorption of the extrusion chromatin is accomplished before the start of the second fission.

The second division is initiated, as in the first, by the activity of the micronucleus. All stages appear to be the same as in the preceding division (Plate I, Figs. 18 and 19) with the result that four daughter ciliates are formed within the original division cyst membrane. Occasionally the cyst wall becomes soft and irregular and liberates the two daughters before the second division. Figure 17 represents a case of binary fission just before the liberation of the daughter cells. The extrusion chromatin has not been absorbed yet, and may not be until after the daughters become free-swimming organisms.

Following the second cell division each macronucleus again undergoes a reorganizational process whereby more extrusion chromatin is formed and cast out into the cytoplasm (Plate I, Figs. 20 and 21).

After this process has taken place the four daughter ciliates become more active, the cyst wall becomes softer and more irregular and finally ruptures. In the majority of cases enough time elapses during the freeing process for the extrusion chromatin to become absorbed but sometimes the cyst membrane ruptures early and the young daughter organisms each carry with them the remains of the residual ball (Plate I, Fig. 23). This residual ball might easily be mistaken for a supernumerary micronucleus, as we feel sure has been the case in the report of Penn (1937) on the occurrence of more than one micronucleus.

When, as occasionally happens, as mentioned above, binary or quadruple division takes place without the formation of a definite cyst membrane there occurs exactly the same nuclear activity as found in the normal division cyst. Chromatin extrusion follows each division in as regular and predictable a fashion as that just described. Figure 22 represents the end result of a quadruple division without cyst formation. These daughter ciliates are completely reorganized and all trace of the residual chromatin has disappeared. We have been able to find all stages representing the above process of chromatin elimination in these divisions but because of the duplication of the conditions found during the normal process and because division without cyst formation was the exception in our material we felt that illustrations would be redundant.

The above process of the alternation of a free-swimming, feeding organism with reproduction within the division cyst is repeated every eight hours, on the average, either in isolation or mass cultures so long as fresh medium is provided. Whether or not there will be found a waning vitality over extended periods of time we cannot tell at present. Experiments testing this point are being carried out and will be reported at a later date.

When conditions within the culture change due to the accumulation of waste products resistant cysts are formed. This process is accomplished in a very short period of time and involves the laying down of a thick, relatively impermeable wall, the absorption of the food bodies and the concentration of the cytoplasm. No activity on the part of the micronucleus is observed but the macronucleus proceeds to the most profound reorganization as yet reported for any holotrichous ciliate.

Resistant cyst formation in a trophic organism proceeds with a diminution in size and a rounding up of the ciliate. As the heavy cyst wall is secreted the cilia disappear and the cyst becomes firmly attached to the substrate. The nuclear apparatus is much the same in appearance as in the trophic stage except for the fact that both

nuclei become smaller and slightly more dense (Plate II, Fig. 24). The food inclusions are rapidly absorbed and the cytoplasm then contains numerous minute refringent bodies (Plate II, Figs. 25 and 26). After the disappearance of the food balls but before the disappearance of the refringent bodies the macronucleus begins to elongate and to differentiate into two distinct regions. One end becomes more intensely staining than the other as if the chromatin was becoming com-

Explanation of Plates

All figures are of *Colpoda cucullus*, and with the exception of Figs. 4–8 the magnification is × 1,000. Figs. 4–8 represent a magnification of × 2,000. All figures are from preparations treated with the Feulgen reagent. Fixatives used were Schaudinn's fluid with 5 per cent acetic acid for the preparations illustrated on Plate I and Gilson-Carnoy fluid for the illustrations on Plate II. The cilia, which are present in all stages represented on Plate I, have been omitted from the illustrations. Asterisks (*) represent residual chromatin.

Plate I

Explanation of Figures

Fig. 1. Trophic ciliate showing typical nuclear apparatus, food inclusions, contractile vacuole and cytoplasmic vacuoles. Note the plaque-like arrangement of the macronuclear chromatin.

Fig. 2. Ciliate about to undergo encystment prior to reproduction. The macronuclear chromatin has become dispersed and flocculent.

Fig. 3. Early division cyst. Micronucleus enlarged and the macronuclear chromatin beginning to collect in aggregates.

Figs. 4–8. Representative micronuclei during mitosis.

Fig. 9. Micronucleus in metaphase and macronuclear chromatin in the form of irregular aggregates.

Fig. 10. Later stage. Macronucleus elongating.

Fig. 11. Daughter micronuclei at the poles of the elongated macronucleus.

Fig. 12. Constriction of the macronucleus.

Fig. 13. Plasmotomy completed and the two daughter macronuclei rounded up. No indication of chromatin differentiation for elimination as yet.

Fig. 14. Budding off of extrusion chromatin (*).

Fig. 15. Slightly later stage.

Fig. 16. About the same condition as the previous stage and a timed preparation. This cyst represents the first one formed after emerging from the resistant cyst. Note small size.

Fig. 17. Probably a case of binary fission with the two daughter ciliates about to leave the wrinkled cyst membrane. Note extrusion chromatin (*).

Fig. 18. Prophase of the second division. The extrusion chromatin has been absorbed in the cytoplasm.

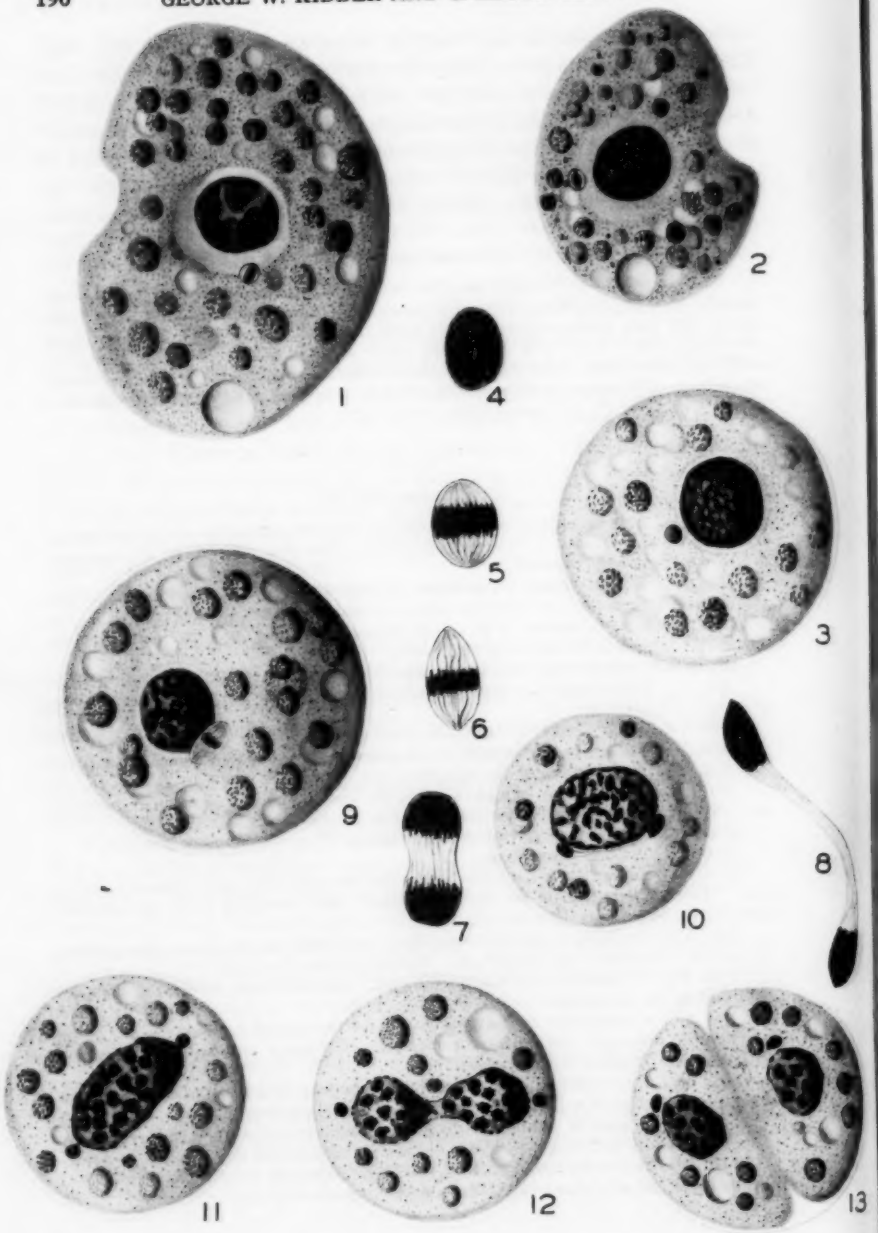
Fig. 19. Constriction of the macronuclei for the second division.

Fig. 20. Second cell division completed. Extrusion chromatin being given off from each daughter macronucleus.

Fig. 21. Four small ciliates about to emerge from the division cyst. Within the cytoplasm of each will be seen the residual ball of chromatin (*).

Fig. 22. A case of quadruple division without encystment. This represents the last stage with the daughter ciliates completely reorganized and about to separate.

Fig. 23. A free-swimming ciliate just after emerging from the division cyst. The residual ball of chromatin (*) has not been absorbed as yet.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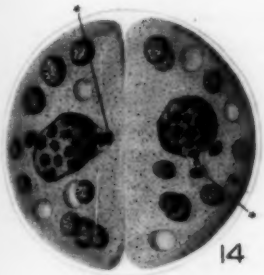
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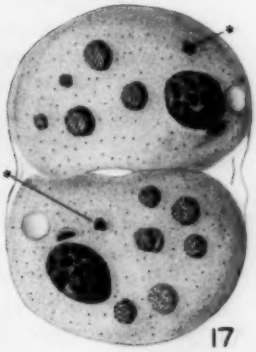
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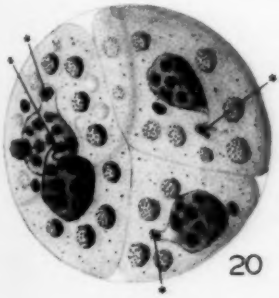
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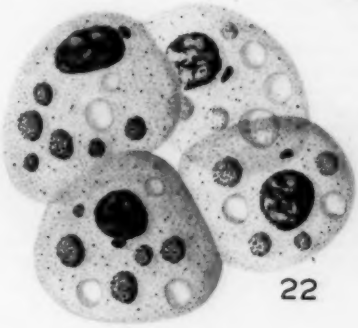
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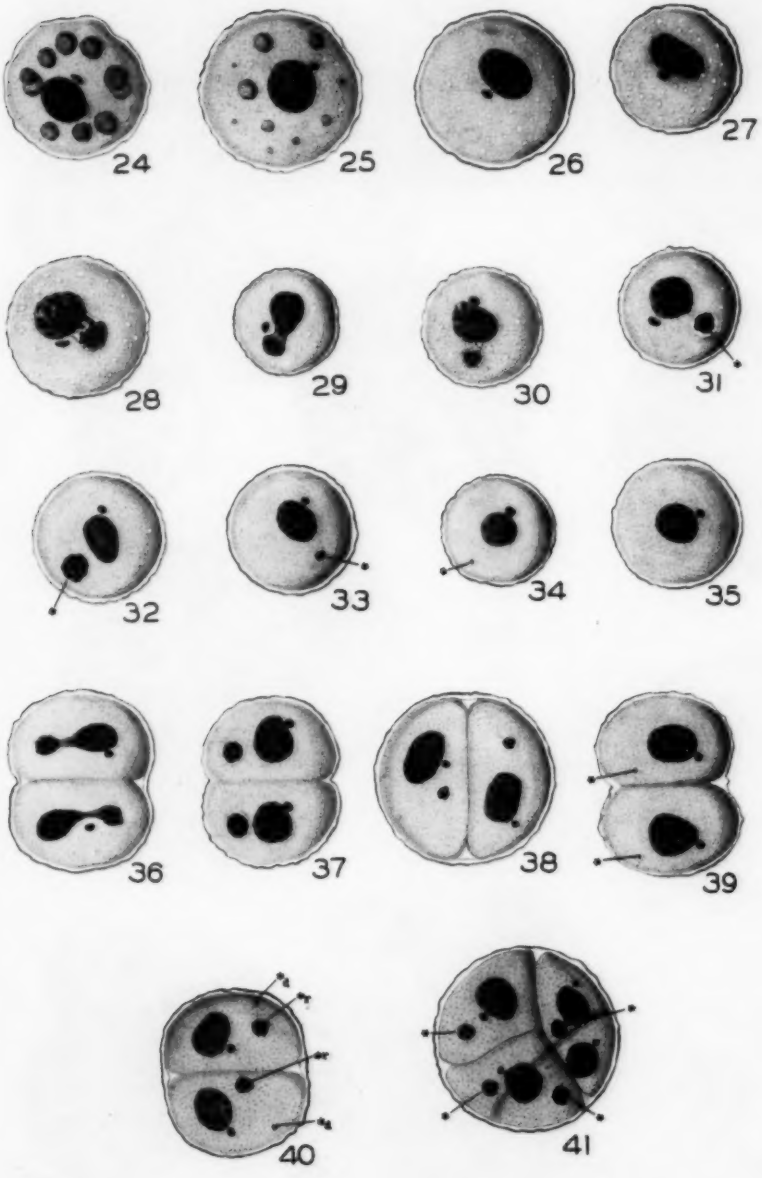
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pacted. This compacted region varies in size from one-third to one-half the whole nucleus (Plate II, Fig. 27). Very rapidly the compacted area buds off from the rest of the macronucleus leaving its chromatin in an irregular granular condition (Plate II, Fig. 28). The compact bud moves farther away from the nucleus until the connection between the two severs and the deeply-staining chromatin rounds up in the cytoplasm (Plate II, Figs. 29, 30 and 31). The amount of chromatin extruded varies considerably in the different cysts. In extreme cases it forms a ball as large as the remaining reorganized macronucleus but usually it is somewhat smaller (Plate II, Figs. 31 and 32). The ultimate resorption of the cast-out chromatin takes place by a gradual diminution in size but no apparent diminution in staining capacity (Plate II, Figs. 33 and 34). Within a few hours after encystment the extruded chromatin has disappeared and there is no further activity within the cyst until excystment. The refringent bodies have gradually disappeared during this process of nuclear reorganization and the resting cyst then possesses a very clear, slightly granular cytoplasm in which the micro- and macronucleus are embedded (Plate II, Fig. 35).

PLATE II

FIG. 24. Newly-formed resistant cyst characterized by the possession of food spheres.

FIG. 25. Later stage showing the diminution of food bodies and the concomitant appearance of the small, refringent bodies.

FIG. 26. The food bodies have entirely disappeared and the refringent bodies are numerous.

FIG. 27. Early stage in the differentiation of the extrusion chromatin within the macronucleus. The extrusion chromatin stains more intensely than the chromatin which is destined to remain.

FIGS. 28–30. The budding off of the ball of extrusion chromatin.

FIG. 31. The connection between the extrusion bud and the macronucleus has broken. Note the globular condition of the large extrusion mass (*).

FIG. 32. A large extrusion mass (*) somewhat later than the preceding stage.

FIGS. 33–34. The extrusion chromatin mass (*) diminishes in size. Note also that the refringent bodies within the cytoplasm have disappeared.

FIG. 35. A reorganized resistant cyst with compact clear cytoplasm and a smoothly granular, compact macronucleus. In this condition the resistant cyst will remain until the cultural conditions are altered sufficiently to induce its excystment.

FIGS. 36–39. Chromatin elimination from the macronuclei of two-cell resistant cysts duplicating, in each cell, the conditions seen in the single-celled resistant cyst.

FIG. 40. Two-celled resistant cyst in which cyst formation occurred before the extrusion chromatin of the divisional reorganization was completely absorbed. The divisional extrusion chromatin is represented by the small, deeply staining balls (*d) while the resistant cyst extrusion chromatin is represented by the large masses (*r).

FIG. 41. Four-cell resistant stage showing typical extrusion chromatin in each cell. This type of cyst is relatively rare.

FIG. 42. Small, clear, cyst-like structures which appeared in a few old liquid cultures and which are thought to represent degenerate resistant cysts.

We have found a few cases where two buds of waste chromatin have been extruded from the reorganizing macronucleus but these are rare.

When, as often happens, division cysts become resistant cysts there is a complete reorganization within each of the daughter ciliates identical with that of the single-celled cyst described above. Usually there appears to be enough time for the divisional reorganization of the macronucleus to proceed to completion and the subsequent resorption of the extrusion chromatin to take place before resistant cyst macronuclear reorganization sets in (Plate II, Figs. 36, 37, 38, 39 and 41). Rarely are there found resistant cysts where both divisional extrusion chromatin and resistant cyst extrusion chromatin are present (Plate II, Fig. 40). Also resistant cysts containing two cells are much more frequent in occurrence than those containing four cells.

Discussion

In our opinion the actuality of macronuclear reorganization involving the elimination of residual chromatin may possibly be demonstrated universally among the holotrichous ciliates. The definite establishment of the elimination of residual chromatin as a single ball during divisional reorganization has been made in the following forms: *Kidderia* (*Conchophthirius*) *mytili* (Kidder, 1933*a*), *Ancistruma isseli* (Kidder, 1933*b*), *Conchophthirius anodontæ*, *C. curtus*, *C. magna* (Kidder, 1934), *Myxophyllum* (*Conchophthirius*) *steenstrupii* (Rossolimo and Jakimowitsch, 1929), *Allosphærium convexa* (Kidder and Summers, 1935) and *Urocentrum turbo* (Kidder and Diller, 1934). A number of other species undoubtedly fall into this group if we can judge by the published reports (*see Loxocephalus*, Behrend, 1916 and *Eupoterion pernix*, MacLennan and Connell, 1931). Post-divisional chromatin elimination, i.e. the casting into the cytoplasm of residual chromatin from each of the daughter macronuclei after separation, is known to occur in *Ichthyophthirius multifiliis* (Haas, 1933), *Colpidium colpoda*, *C. campylum* and *Glaucoma scintillans* (Kidder and Diller, 1934), *Chilodonella labiata* and *C. faurei* (MacDougall, 1936).

In all the cases cited above the chromatin to be eliminated is differentiated within the macronucleus prior to its division and thus advertises itself. All species, therefore, in which no differentiation into regions occur prior to fission have been described as having a "clean" macronuclear division. As the vast majority of ciliates divide without encystment, and the two daughter cells separate immediately after fission, this phase of their cytology has been neglected. It seems entirely possible to us that if attention were paid to the young daughter

cells after fission the occurrence of macronuclear reorganizational processes would be discovered in a great many if not all species. Another possibility that suggests itself is that the many cases of chromatin-like fragments in the cytoplasm so often reported in ciliate studies may be explained by some process of macronuclear elimination during reorganization. We wish to emphasize the necessity for more thorough and critical cytological work with this problem in mind to determine whether or not *macronuclear reorganization with chromatin elimination will be found to be a universal principle applicable to all holotrichous ciliates.*

As to the exact significance of this regular though complicated process, we are still unable to say. It has been suggested (Kidder, 1933*a*, 1933*b*, 1934; Kidder and Diller, 1934) that the eliminated chromatin represents worn-out material and the process might be a cleaning out of the macronucleus toward a perfect organization. It was further suggested (Kidder and Diller, 1934) that the profound reorganization which occurs at every division of *Colpidium colpoda*, *C. campylum* and *Glaucoma scintillans* might account for the fact that conjugation rarely occurs in these species, the reorganizational process serving to restore the cells to their fundamental condition and thereby decreasing the necessity for conjugation. The above suggestion seems to us to apply as well as any other to *Colpoda cucullus.*

Concerning the drastic reorganization that occurs immediately after the resistant cyst is formed, it seems logical to suppose that this represents the ridding of the macronucleus of materials no longer needed in the state of decreased or suspended metabolism. Materials accumulated in the macronucleus through the very activity of encystment may be detrimental to the resting cell or to the process of excystment, to come at a later date. Unfortunately we have very few records of what goes on within the resistant cysts of the various species of ciliates with which to make comparisons. We know from a few sources (see Tittler, 1935) that a process of endomyxis sometimes accompanies encystment, whereby the old macronucleus is completely discarded and a new one formed from micronuclear material. These cases would seem to represent simply a different method for accomplishing the same general result as occurs in *Colpoda cucullus*, the production of a purified macronucleus.

That the reorganizational process occurring within the resistant cyst takes the place of the divisional reorganization of the macronucleus is denied by direct observation. In the very first division after emergence from the resistant cyst the normal reorganizational chromatin elimination occurs. This was determined by timed preparations

and Figure 16 illustrates a first division cyst. The small size is usual as the ciliates emerging from the resistant cysts are very small and usually reproduce before full growth is attained, a condition noted in the case of *Tillina magna* by Gregory (1909). The time factor may play an important rôle here, however, as there is the possibility of the aging of the macronucleus during its long period within the resistant cyst, resulting in the necessity for reorganization immediately upon again taking up an active life.

The actuality of a regular and predictable macronuclear reorganization with the elimination of quantities of chromatin during division and within the resistant cyst has been established for *Colpoda cucullus* but a completely satisfactory explanation of its significance awaits further investigation. Experiments are now under way which, it is hoped, will throw some light on this question.

Summary

1. A complete description of the nuclear activity of *Colpoda cucullus* Muller is given for the first time.

2. In our strain the normal method of reproduction takes place within a thin cyst membrane. Usually two divisions result giving rise to four daughter organisms which break out of the cyst and repeat the process. Occasionally binary fission occurs within the cyst. Rarely quadruple division occurs without encystment, as described by Penn (1937).

3. Following each cell division there occurs a reorganizational process within the daughter macronuclei resulting in the elimination of a quantity of residual chromatin. The residual chromatin is cast into the cytoplasm where it is absorbed. Elimination of residual chromatin is regular and synchronous in each cell whether the division has occurred within a cyst or not.

4. When cultural conditions are poor resistant cysts are formed.

5. The resistant cysts are formed by the secretion of a heavy cyst membrane, the absorption of the food inclusions and the concentration of the whole protoplasmic mass.

6. Immediately following the formation of the resistant cyst membrane the macronucleus undergoes a profound reorganization during which a variable, but always a considerable amount of chromatin is budded off and cast into the cytoplasm where it is absorbed. No micronuclear activity occurs at this time.

7. The question of chromatin elimination from the macronuclei of holotrichous ciliates is discussed and the opinion expressed that this phenomenon may represent a universal principle.

LITERATURE CITED

BARKER, H. A., AND C. V. TAYLOR, 1931. A study of the conditions of encystment of Colpoda cucullus. *Physiol. Zoöl.*, **4**: 620.

BEHREND, K., 1916. Zur Conjugation von Loxocephalus. *Arch. f. Protist.*, **37**: 1.

BODINE, J. H., 1923. Excystation of Colpoda cucullus. *Jour. Exper. Zoöl.*, **37**: 115.

CALKINS, G. N., 1930. Uroleptus halseyi Calkins. II. The origin and fate of the macronuclear chromatin. *Arch. f. Protist.*, **69**: 151.

ENRIQUES, P., 1908. Sulla morfologia e sistematica del genere Colpoda. *Arch. Zool. Exper. et Gén.*, **8**: (N. & R.) 1.

FEULGEN, R., AND H. ROSSENBECK, 1924. Mikroscopisch-chemischer Nachweis etc. *Zeitschr. Physiol. Chem.*, **135**: 203.

GOODEY, T., 1913. The excystation of Colpoda cucullus from its resting cysts, and the nature and properties of the cyst membranes. *Proc. Roy. Soc. London*, (B) **86**: 427.

GREGORY, L. H., 1909. Observations on the life history of Tillina magna. *Jour. Exper. Zoöl.*, **6**: 383.

HAAS, G., 1933. Beiträge zur Kenntnis der Cytologie von Ichthyophthirius multifiliis Fouq. *Arch. f. Protist.*, **81**: 88.

KAHL, A., 1931. Die Tierwelt Deutschlands. 21 Teil: Protozoa. Fischer, Jena.

KIDDER, G. W., 1933*a*. Studies on Conchophthirius mytili de Morgan. I. Morphology and division. *Arch. f. Protist.*, **79**: 1.

KIDDER, G. W., 1933*b*. On the genus Ancistruma Strand (Ancistrum Maupas). I. The structure and division of A. mytili Quenn. and A. isseli Kahl. *Biol. Bull.*, **64**: 1.

KIDDER, G. W., 1934. Studies on the ciliates from fresh water mussels. II. The nuclei of Conchophthirius anodontae Stein, C. curtus Engl., and C. magna Kidder, during binary fission. *Biol. Bull.*, **66**: 286.

KIDDER, G. W., AND W. F. DILLER, 1934. Observations on the binary fission of four species of common free-living ciliates, with special reference to the macronuclear chromatin. *Biol. Bull.*, **67**: 201.

KIDDER, G. W., AND F. M. SUMMERS, 1935. Taxonomic and cytological studies on the ciliates associated with the amphipod family Orchestiidæ from the Woods Hole district. *Biol. Bull.*, **68**: 51.

MACDOUGALL, M. S., 1936. Étude cytologique de trois espèces du genre Chilodonella Strand. Morphologie, Conjugaison, Réorganisation. *Bull. Biol. France et Belg.*, **70**: 308.

MACLENNAN, R. F., AND F. H. CONNELL, 1931. The morphology of Eupoterion pernix, gen. nov., sp. nov.: a holotrichous ciliate from the intestine of Acmæa persona Eschscholtz. *Univ. Calif. Publ. Zoöl.*, **36**: 141.

PENN, A. B. K., 1937. Reproduction in Colpoda cucullus. *Arch. f. Protist.*, **88**: 366.

RHUMBLER, L., 1888. Die verschiedenen Cystenbildung und die Entwicklungsgeschichte der holotrichen Infusoriengattung Colpoda. *Zeitschr. f. wissensch. Zool.*, **46**: 549.

ROSSOLIMO, L. L., AND FRAU K. JAKIMOWITSCH, 1929. Die Kernteilung bei Conchophthirius steenstrupii St. *Zool. Anz.*, **84**: 323.

STEIN, F., 1854. Die Infusionsthiere auf ihre Entwicklung untersucht. Leipzig.

TITTLER, I. A., 1935. Division, encystment and endomyxis in Urostyla grandis with an account of an amicronucleate race. *La Cellule*, **44**: 189.

TURNER, J. P., 1937. Studies on the ciliate Tillina canalifera n. sp. *Trans. Am. Microsc. Soc.*, **56**: 447.

WENYON, C. M., 1926. Protozoölogy. New York.

EMBRYONIC DETERMINATION IN THE ANNELID, SABELLARIA VULGARIS

I. The Differentiation of Ectoderm and Endoderm When Separated through Induced Exogastrulation

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Introduction

The method of transplantation has brought to light the abilities of certain embryonic parts to induce, or organize, the development of other embryonic structures in adjacent tissues. The use of this technique has, however, only recently been extended to the so-called "mosaic" eggs. Tung (1934) reported experiments involving the fusion of eggs at the two-cell stage, fusion of micromeres and macromeres, and rotation of micromeres in the eggs of *Ascidiella*. He found no deviation from the normal in the development of the cells giving rise to notochord and muscle. However, there was evidence that the sense organ and the ectodermal adhesive organ were induced. Two years later, there appeared abstracts by Hörstadius (1936) and by the writer (1936) describing the development of transplanted blastomeres of *Cerebratulus* and *Sabellaria* respectively. Hörstadius (full report, 1937) found no evidence of induction in fusions of various quartets of the 16-cell stage. The essentially similar results of the writer will be described in greater detail in the second paper of the present series.

It is the purpose of this first paper to describe the results of an experiment which, though similar to isolation experiments, possesses certain of the added advantages of the transplantation method. The experiment consists of the production of larvæ in which the endoderm cells grow out, during gastrulation, from their normal position underlying the ectoderm. This affords a way of testing the independent differentiation of the two layers and, by inference, the effect of one layer upon the other during normal development.

Material and Methods

The egg used is that of the marine annelid, *Sabellaria vulgaris*. That this is an egg of the classical mosaic type is shown by the completely partial development of isolated blastomeres (Hatt, 1932). The

procedure for obtaining fertilized eggs of this species has been described elsewhere (Novikoff, 1937). The larvæ of this species are closely similar to those described by Wilson (1929) for the two European species, *alveolata* and *spinulosa*.

Exogastrulae can be produced in considerable numbers by a treatment which removes the vitelline membranes of fertilized ova. A variation of the method employed by Hatt (1931) has proved to be quite effective. At any time before they have begun to change their form in preparation for cleavage, the eggs are washed once with an isotonic NaCl solution brought to pH 9.6 by the addition of Na_2CO_3 (.98 gram in 1 liter of solution) and are then placed into 5 cc. of the same solution. The eggs adhere to each other in this medium, forming large aggregates, but as the egg membranes disappear, the aggregates break up. When this has occurred, 5 cc. of acidified NaCl solution (0.45 cc. 1.0 N HCl in 100 cc. 0.53 N NaCl) are added, bringing the medium to pH 8.2. When the eggs have settled to the bottom of the dish, they are washed once in sea water and transferred to a Syracuse dish containing fresh sea water. Among the swimming larvæ which develop, the exogastrulæ can readily be distinguished from the normal larvæ in the dish.

Representative stages in the development of exogastrulæ and control larvæ are described in the text. All text figures are camera lucida drawings of living larvæ; the description accompanying each figure refers not to that specific larva but is a general description of larvæ at that stage in development. The time intervals given are approximate, for temperatures ranging from 19° to 24° C.

Description of Results

It is possible to follow the details of ectodermal and endodermal differentiation in living larvæ. In the normal course of development the ectoderm gives rise to the following structures: a prototroch of long active cilia, at first encircling the animal but later incomplete on the dorsal surface; chromatophores, which are yellow when they form but later change to green; an apical tuft which disappears when replaced by short non-motile cilia; paired chætæ-sacs from which extend long serrated bristles; a single, short, non-motile posterior cilium; several long, non-motile dorsal cilia in the region of the prototrochal gap; a single orange-red eye spot on the left side, towards the dorsal surface; and rapidly moving cilia ("neurotroch") in a ventral depression leading to the mouth cavity of the fully formed trochophore. The endoderm differentiates into a tripartite gut consisting of œsophagus, stomach, and intestine. The œsophageal cells are lined in-

ternally with many long actively-moving cilia. The stomach and intestine are also ciliated but the motion of their cilia differs from that of the œsophagus; they move more slowly and remain more rigid during the effective stroke.

In order to compare the development of the eggs from which the membranes have been removed with that of the normal eggs, a series of stages in the development of both are described. Those stages have been chosen which show definite advances in structural differentiation.

The early cleavages of the membraneless eggs are similar in all respects to those of normal eggs. Although the details of the later cleavages have not been described for this species, any marked difference in this process between the two types of eggs would have been noted. But no such variations were apparent. The first noticeable departure from normal development appears at the time when the larvæ show the first signs of movement. This is usually between six and seven hours after fertilization and probably corresponds to the time when gastrulation is normally occurring.

Comparison of Complete Exogastrulæ with Normal Larvæ

Six to Seven-hour Larva.—Normal (Fig. 1*A*). There is no regularity in the shape of the larvae at this time; it may vary from a spherical to a roughly conical form. The vitelline membrane, retaining the many wrinkles characteristic of the earlier stages in development, is still far removed from the body of the larva. Through the membrane, there project a group of long cilia at the future anterior end of the larva, and many short, rapidly moving cilia which form a narrow girdle completely encircling the larva.[1] These cilia constitute the apical tuft and the prototroch respectively. The larva is rather opaque and it is with difficulty that the large internal cells can be distinguished. Directed towards the inner cells and situated slightly posterior to the prototroch is the blastoporal indentation.

Exogastrula (Fig. 1*B*). These larvæ are more uniformly regular than the normal larvæ of the same age. Not obscured by the vitelline membrane as they are in the normal larvæ, the cilia of the apical tuft and prototroch are more easily distinguished. The distance between the apical end and the prototroch (i.e., the pretrochal region) is the same as it is in the normal larva, but the post-trochal region is considerably lengthened by a posterior outgrowth of cells. No blastopore is present.

Thirteen-hour Larva.—Normal (Fig. 1*C*). The larva has now rounded out and there are only slight irregularities in the surface.

[1] The cilia in the early larvæ may be brought out more clearly with darkfield illumination.

It has elongated somewhat in the antero-posterior direction and is just beginning to assume the typical trochophore appearance. The apical tuft is directed forward as the larva swims, rotating on its longitudinal axis. The prototroch, which formed a complete ring about the animal at the preceding stage, is interrupted by a short region with no cilia; this gap marks the dorsal surface of the trochophore. At the posterior, broader end there projects a fine non-motile cilium. The surface of the larva now has a general gold-yellow coloration when viewed by transmitted light. Through the outer ectoderm cells the "anlage" of the gut is discernible. From a crescent-shaped cavity at the apical end, a narrow canal leads through the gut cells to open on the surface at the posterior end. The blastopore, which has become progressively smaller up to the eleventh hour of development, has completely disappeared.

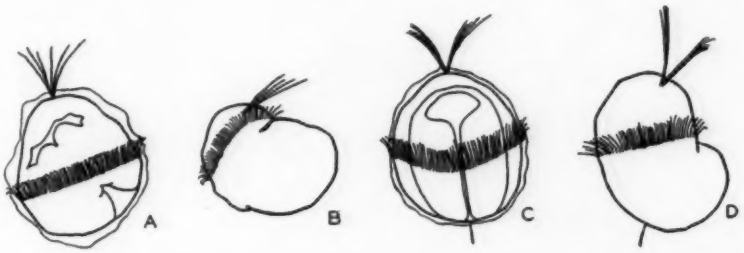

FIG. 1. *A*. Normal larva. Seven hours after fertilization. Shows apical tuft and prototroch. Posterior to the prototroch is the blastoporal indentation. *B*. Exogastrula. Seven hours. Viewed from posterior end. Shows apical tuft, prototroch, and elongate post-trochal region. *C*. Normal larva. Thirteen hours. Shows apical tuft, prototroch, posterior cilium, and differentiating gut. *D*. Exogastrula. Thirteen hours. Shows apical tuft, prototroch, posterior cilium, and elongate post-trochal region.

Exogastrula (Fig. 1*D*). This larva is considerably more elongate, antero-posteriorly, than the normal larva of the same age. The structure of the apical tuft, prototroch, and posterior cilium is normal, and the apical tuft is in the usual location. On the other hand, the relative positions of both the prototroch and the posterior cilium are strikingly different. The former is situated more anteriorly in the larva; the latter is to one side of the larva and projects laterally instead of posteriorly. The general yellow coloration of the embryo does not extend very far beyond the equator; the cells at the posterior end are colorless. The crescent-shaped cavity and the longitudinal canal present in the endoderm cells of the normal larva at this stage are not present.

Eighteen-hour Larva.—Normal (Figs. 2*A* and *B*). Since the preceding stage, the larva has become more spherical and more regular in outline. The membrane, almost free of irregularities, is more closely applied to the larval surface. The cilia of the apical tuft are now considerably longer and they move slowly in two distinct groups. The prototrochal gap has grown in size; the posterior cilium is still present, and the yellow pigment has become localized into a number of larger areas on the surface of the body. The endoderm cells within are now assuming the form of the gut; one can recognize the three main divisions—the ventral œsophagus runs anteriorly into the wider stomach, which in turn is continuous with the intestine. The only lumen visible in the gut is the narrow canal described in the earlier larva; it runs through the stomach and intestine and opens as the anus. Slightly anterior to the prototroch and on either side of the

FIG. 2. Larvæ, eighteen hours after fertilization. *A*. Normal larva. Side view. Shows apical tuft, prototroch, gut, and pigmented areas. *B*. Normal larva. Dorsal view. Shows prototrochal gap, stomach and intestine portion of gut, and posterior cilium. *C*. Exogastrula. Post-trochal region is elongated and hollow.

stomach there has developed a spherical structure, the chæta-sac, from which the long bristles later grow.

Exogastrula (Fig. 2*C*). Changes similar to those described for the normal larva have occurred in the apical tuft, prototroch, and pigmentation of the exogastrula. The apical tuft is composed of two groups of long cilia. The prototrochal gap extends over the dorsal surface. The yellow pigment has become more localized, but since the pigment was originally restricted to the more anterior part of the larva, the posterior portion is completely colorless. The difference between this larva and the normal one of the same age is, however, much more pronounced than at the preceding stage. In contrast to the fairly spherical form of the normal, it is narrow and long. It possesses no internal gut and in the posterior half there appears a large cavity. The posterior cilium is again laterally displaced.

Twenty-four-hour Larva.—Normal (Figs. 3*A* and *B*). Important changes have occurred between this and the preceding stage of development. In the apical region there have appeared, alongside the apical tuft, a number of short, non-motile cilia. On the dorsal surface of the larva, in the region of the enlarged prototrochal gap, there are two or three long non-motile cilia. The posterior cilium is still short and non-motile. The large pigmented areas have been broken up into well-defined yellow chromatophores. The gut has acquired a continuous lumen, lined with rapidly moving cilia. The cilia of the œsophagus differ from those of both the stomach and intestine in that they are slightly longer, are more numerous, per unit surface area, and move more rapidly. In their movement, they are bent more than are the stomach-intestinal cilia. The œsophagus opens to the exterior through

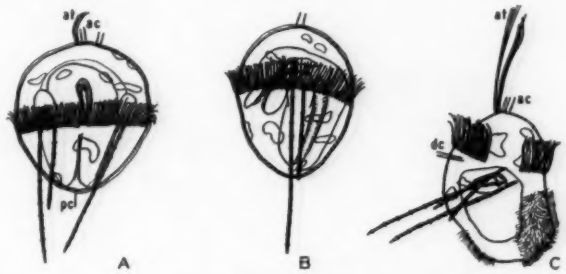

FIG. 3. Larvæ, twenty-four hours after fertilization. Abbreviations: *at*, apical tuft; *ac*, apical cilia; *pc*, posterior cilium; *dc*, dorsal cilia. *A*. Normal larva. Ventral view. Mouth, œsophagus, and intestine visible. The pigmented areas are broken up into well-defined chromatophores. Two pairs of bristles are present. *B*. Same larva as *A*. Side view. *C*. Exogastrula. Shows elongate, hollow, post-trochal region. On the surface, toward the posterior end, short cilia are visible.

the mouth, the intestine through the anus. From each of the chætæ-sacs there now extend two bristles. The position and structure of these bristles are very characteristic. Arranged at intervals along their length are serrated "collars." As the larva swims about, the bristles, projecting posteriorly, are seen being rotated about the long axis of the animal. That muscles have developed can be deduced from the observed contraction of the larvæ and the movement of the chætæ-sacs and bristles.

Exogastrula (Fig. 3*C*). At this stage, the exogastrulæ can easily be distinguished, even with low magnification, from the normal animals. The most striking feature is the behaviour of the bristles when the animal is in motion. The larva rotates as usual as it moves in a forward direction. But the bristles project laterally instead of

posteriorly, and they are accordingly turned over and over in a wide arc, rather than rotated on the longitudinal axis of the larva. Viewing the animal from above, the bristles therefore appear and disappear from sight. No gut is present, and the cavity of the preceding stage has increased noticeably in size. On the surface of the posterior portion of the larva there are now rapidly beating cilia, of the same size as the cilia in the gut of the normal larva. They are usually found in two distinct extensive areas, with the larger of the two areas covered with cilia characteristic of the normal œsophagus. It is possible to distinguish the single asymmetrically-placed posterior cilium. The larvæ show the following changes over the preceding stage, which are similar to the changes in normal development: (1) the appearance of short, non-motile cilia at the apical end; (2) the formation of smaller, yellow chromatophores, although in the exogastrulæ they are

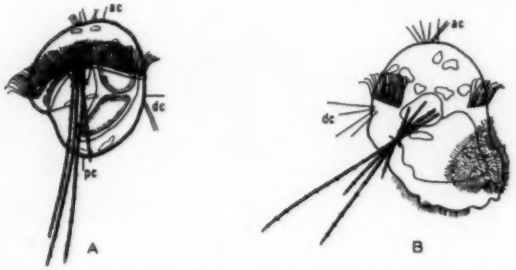

FIG. 4. Larvæ, twenty-nine hours after fertilization. Abbreviations as in Fig. 3. *A*. Normal larva. Side view. Mouth, stomach, and intestine are well-formed. The bristles on the left side only have been drawn. *B*. Exogastrula (slightly flattened). Dorsal view. Only four of the eight bristles have been drawn.

limited to little more than the anterior half of the larva; (3) the development of dorsal cilia near the gap in the prototroch; (4) the appearance of contractions of the larva, albeit these contractions are considerably weaker than those of the normal larva; (5) the formation of two pairs of bristles of the same notched structure as described for the normal.

Twenty-nine-hour Larva.—Normal (Fig. 4*A*). The larva is beginning to assume the appearance of the fully-developed trochophore. The membrane is closely applied to the surface of the larva. The apical tuft has disappeared, and only the non-motile cilia remain at the anterior end. The prototroch is raised slightly, forming the so-called "hood fold" (Wilson, 1929). The single posterior cilium persists. The small yellow chromatophores are taking on a green tinge. The stiff dorsal cilia are longer and more numerous than in the previous

stage. The œsophagus, lined with many active cilia, leads into the stomach. The stomach is partially separated from the intestine by an incomplete shelf; the intestine is lined with cilia of the type found in the stomach. The number of bristles varies from three to four pairs. On stimulation (i.e., when the slide or the dish is tapped gently), the larva rapidly contracts and the bristles are spread into a fan-like arrangement. After several seconds in this position, the animal returns to its usual form.

Exogastrula (Fig. 4*B*). The general appearance of the larva is similar to that of the preceding stage. The three or four pairs of bristles project laterally and, as the animal moves, are rotated in the manner described above. On stimulation, they are spread out as in

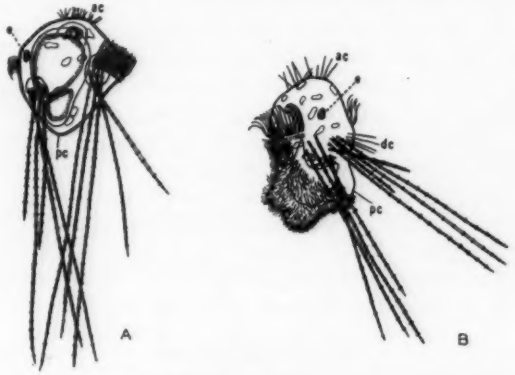

FIG. 5. Larvæ, forty-eight hours after fertilization. Abbreviations as in Fig. 3. *A*. Normal larva. Dorsal view. To the left of the stomach is the eye spot (*e*). *B*. Exogastrula. Dorsal view. A large part of the post-trochal region is covered with short cilia.

the normal larva, although to a lesser extent. There is no internal gut, and a large cavity is present inside the larva. The chætæ-sacs project into the cavity. As in the normal larva of this age, only the apical cilia persist at the anterior end. The dorsal cilia have elongated and increased in number. The chromatophores, limited to about the anterior half of the larva, are becoming greener in color. The cilia on the exterior of the post-trochal region are found in two regions. Of these only the ventral region has the cilia of the œsophageal type.

Forty-eight-hour Larva.—Normal (Fig. 5*A*). This is a well-developed trochophore. The post-trochal region has lengthened somewhat. The apical cilia are numerous and the number of dorsal cilia has also increased. There is a single posterior cilium. A second, posterior row

of short cilia has appeared on the hood fold, just posterior to the long prototrochal cilia. On either side of the mouth there has developed a fold covered externally with cilia, the lip fold. The chromatophores have a more pronounced green coloration. On the left side of the larva, near the stomach, there has appeared a single eye spot, composed of closely packed orange-red granules. The gut is clearly differentiated into œsophagus, stomach, and intestine. The stomach has increased in size and has become more spherical. Running posteriorly from the mouth, in a groove along the ventral surface, are the long cilia which constitute the neurotroch. A larva at this time ordinarily has four pairs of bristles, which project posteriorly.

Exogastrula (Fig. 5*B*). Changes similar to those occurring in the normal larvæ have taken place between this and the preceding stage. The apical cilia have become more numerous. The prototroch is better developed and a row of short cilia has been added posterior to it. The dorsal cilia have increased in number and size, and the posterior cilium is single and dorsal. On the dorsal surface, anterior to the prototroch, may be seen the eye spot. Four pair of bristles project laterally as do the bristles of the earlier larvæ. There is no internal gut. The cilia characteristic of the œsophagus and the intestine, together with the cilia of the neurotroch and the lip fold, cover the post-trochal region almost completely. Special mention must be made of the eye spot, since it was not seen in all the larvæ examined. It could not be found in thirteen of the ninety-three larvæ studied.

Partial Exogastrulæ

There are always found in the culture dishes containing the membraneless larvæ a few larvæ which are intermediate in structure between those described as complete exogastrulæ and the normal. These larvæ are illuminating for an understanding of the structure of the more extreme type. Figure 6 shows two views of a well-developed trochophore of this type. Not all the structures are visible in the drawings, but the larva possesses every structure found in the normal animals, although these structures have in some cases differentiated in abnormal positions. The most outstanding feature of these larvæ is the gut structure. The stomach and intestine are clearly normal in form, position, and type of ciliation. The intestine opens posteriorly, as usual, through the anus. At the anterior end of the stomach there is a circular opening lined with cilia which move in a manner characteristic of the cilia of the œsophageal-stomach opening in the normal larva. But this opening leads here not to the œsophagus, but to the outer surface of the larva. In fact, there is no internal

œsophagus at all. On the ventral surface of the larva is a long, conical outgrowth, completely covered with long actively-moving cilia which are characteristic of the œsophagus.

Discussion of Results

There are two important points of difference between larvæ which develop from eggs without membranes and normal larvæ. The first is the complete absence of an internal gut and the second the striking deviation from the normal form of the larva. Instead of possessing the fairly spherical shape, they have elongate bodies in which the posterior structures, the posterior cilium and the paired bristles, are dorsally displaced. The distortion of the posterior part of the larva may be accounted for if we assume that the endoderm cells, which differentiate normally inside the ectoderm to form the gut, grow out so that they come to lie on the surface of the larva.

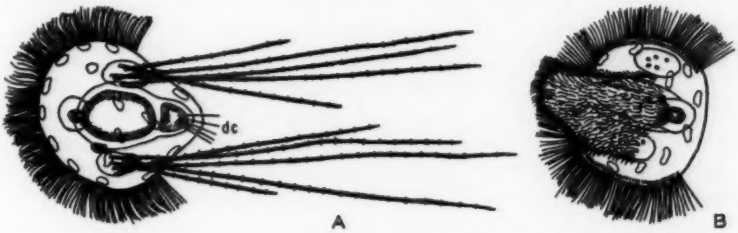

Fig. 6. Partial exogastrulæ. Seventy-two hours after fertilization. *A*. Dorsal view. The opening to the stomach, the stomach, and the intestine are visible. *B*. Ventral view. The bristles have been omitted in the drawing; only their points of origin in the chætæ-sacs are indicated. The opening to the stomach may be seen at the base of the long conical projection covered with short cilia.

That this posterior outgrowth of the endoderm cells has indeed occurred is shown by the following considerations: 1. The gut is completely lacking in these larvæ, and within the post-trochal region of the larva there is a large cavity. 2. The cells situated at the posterior end of these larvæ are not pigmented and never develop chromatophores. In the normal larva the chromatophores are found in the ectoderm; only the endoderm is colorless. 3. In the normal larva, motile cilia do not appear on the external surface of the post-trochal region until approximately thirty-five hours after fertilization. When they do develop, they are restricted to a narrow region, the neurotroch. But in the membraneless larva cilia begin to appear on the surface of the post-trochal region before the end of the first day of development. By twenty-four hours after fertilization they have covered two wide areas. That these cilia are not those of the neurotroch is clear from

the fact that they cover two extensive regions and that they develop twelve hours earlier than do the neurotrochal cilia. Their appearance coincides precisely with the development, normally, of cilia on the inner surface of the endoderm cells. It is also possible to detect differences in the cilia which correspond to the differences between the œsophageal cilia and the cilia of the stomach and intestine.

We may therefore conclude that in these eggs the endoderm has been turned inside out. Whatever the mechanism involved, the end result of this process is comparable to the exogastrulæ that have been described in the sea-urchin (Herbst, 1893; Driesch, 1893) and in the amphibia (Holtfreter, 1933*a*, *b*).

In the partial exogastrulæ, the stomach and intestine develop normally, and only the œsophagus is exogastrulated. And, as we would expect on the basis of our assumption, the general form of the larva is more like the normal than is that of the complete exogastrula.

The most significant feature of exogastrula development in *Sabellaria* is the complete self-differentiation of both endoderm and ectoderm. In the amphibian egg, Holtfreter found that normal differentiation of the ectoderm is dependent upon contact with the mesendoderm. When the latter, instead of coming to lie beneath the ectoderm as it does normally, evaginates, it leaves the ectoderm a wrinkled, hollow sac in which no trace of differentiation into nervous tissue appears. On the other hand, the mesendoderm, although turned inside-out, undergoes self-differentiation; it produces gut, thyroid, pancreas, liver, notochord, musculature, kidney, and gonad. Thus, in the normal course of development, the endoderm differentiates independently of the ectoderm, but the ectoderm must be in contact with the endoderm, or gut roof, to differentiate normally. It is this gut roof which acts in the capacity of an organizer—which induces the formation of a nervous system in the ectoderm.

In *Sabellaria*, on the other hand, there is no deviation from normal differentiation of either the ectoderm or endoderm in the exogastrulæ. The endoderm cells retain their morphological polarity, so that they develop cilia on what is in these larvæ their external surface. And although the tripartite nature of the gut is lost, the type of cilia developed by the exogastrulated œsophagus cells differs from that of the stomach and intestine in the same way as do the normal œsophageal cilia from the normal stomach-intestinal cilia. The ectoderm, too, differentiates normally. The pigment develops and concentrates into yellow areas, which later break up into smaller chromatophores and become distinctly green in color. The apical cilia form at the anterior end and the apical tuft disappears. The serrated bristles develop

normally, although the cells from which they arise have been displaced to one side (dorsally). On the dorsal side there develops the orange-red eye spot. Muscles, as evidenced by contractions of the larva, are also differentiated.

Thus, we find no evidence of any inducing effect of the endoderm upon the ectoderm, or vice versa, in the development of *Sabellaria*. There may possibly be some question concerning the self-differentiation of the eye spot in the exogastrula since in 13 out of 93 cases no eye spot was observed. In more than one hundred normal control larvæ studied, none was found without the eye spot. However, when individual untreated eggs are isolated in small drops of sea water beneath cover-slips it is found that they develop into larvæ in which only the eye spot fails to develop. In these isolates, it is clear that the absence of the eye spot is not due to the morphological absence of an inducing region below the ectoderm. The failure of the ectoderm to form the red pigment may perhaps be due to some general factor such as increased pressure or lower oxygen tension. The same is probably true of those exogastrulæ in which no eye spot is seen.

Thus, the absence of the eye spot in some of the exogastrulæ does not constitute a serious objection to the conclusion that there is no inducing ability of either the ectoderm or endoderm, as tested by their effects upon one another during development. It is possible that in the exogastrulæ the ectoderm has not been completely isolated from the endoderm. The cells which will give rise to the two layers are in contact with each other at one point. Through the study of exogastrulæ alone one can not exclude the possibility of diffusion of materials from one part of one layer across cell boundaries to any part of the other layer; nor can it be denied that the cells may exercise inducing effects upon each other during cleavage. These criticisms merely point to the limitations of this mode of approach. To overcome them, another method may be used—that of transplantation of blastomeres. The results of such experiments in the egg of *Sabellaria vulgaris* form the subject-matter of the second paper in this series.

Summary

1. A simple method for the production of complete and partial exogastrulæ in *Sabellaria vulgaris* is described.

2. In the complete exogastrulæ, no internal gut is formed. In the partial exogastrulæ there is no internal œsophagus, but the stomach and intestine develop normally.

3. Both the ectoderm and endoderm show complete self-differentiation in the exogastrulæ, indicating that one layer exercises no inducing

effect upon the other between six and forty-eight hours of development, from gastrulation to the well-developed trochophore.

It is with pleasure that the writer expresses his gratitude to Professor L. G. Barth for untiring assistance and constant encouragement throughout the course of this investigation.

BIBLIOGRAPHY

DRIESCH, HANS, 1893. Entwicklungsmechanische Studien. VII, Exogastrula und Anenteria. *Mittheil. Zool. Station zu Neapel*, **11**: 221.

HATT, PIERRE, 1931. La fusion expérimentale d'oeufs de "Sabellaria alveolata L." et leur développement. *Arch. de Biol.*, **42**: 303.

HATT, PIERRE, 1932. Essais éxperimentaux sur les localizations germinales dans l'oeuf d'un Annélide (Sabellaria alveolata L.). *Arch. d'Anat. Micros.*, **28**: 81.

HERBST, CURT, 1893. Experimentelle Untersuchungen über der Einfluss der veränderten chemischen Zusammensetzung des umgebenden Mediums auf die Entwicklung der Thiere. 2 Theil. *Mittheil. Zool. Station zu Neapel*, **11**: 136.

HOLTFRETER, JOHANNES, 1933*a*. Die totale Exogastrulation, eine Selbstablösung des Ektoderms vom Entomesoderm. *Arch. f. Entw.*, **129**: 669.

HOLTFRETER, JOHANNES, 1933*b*. Organisierungsstufen nach regionaler Kombination von Entomesoderm mit Ektoderm. *Biol. Zentralbl.*, **53**: 404.

HÖRSTADIUS, SVEN, 1936. Investigations on determination in the early development of Cerebratulus (Abstract). *Biol. Bull.*, **71**: 406.

HÖRSTADIUS, SVEN, 1937. Experiments on determination in the early development of Cerebratulus lacteus. *Biol. Bull.*, **73**: 317.

NOVIKOFF, ALEX B., 1936. Transplantation of the polar lobe in Sabellaria vulgaris (Abstract). *Anat. Rec.*, **67**: (Supplement 1) 57.

NOVIKOFF, ALEX B., 1937. Sabellaria vulgaris. Culture Methods for Invertebrate Animals, by Galtsoff et al. Comstock Publishing Co. Inc., p. 187.

TUNG, TI-CHOW, 1934. Recherches sur les potentialtés des blastomères chez Ascidiella scabra. *Arch. d'Anat. Micros.*, **30**: 381.

WILSON, DOUGLAS P., 1929. The larvæ of the British sabellarians. *Jour. Mar. Biol. Ass'n.*, **16**: 221.

EMBRYONIC DETERMINATION IN THE ANNELID, SABELLARIA VULGARIS

II. Transplantation of Polar Lobes and Blastomeres as a Test of their Inducing Capacities

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Introduction

E. B. Wilson (1904*a*) demonstrated that the egg of the annelid, *Lanice*, belonged to the group of so-called mosaic eggs, since isolated halves of the two-cell stage developed into partial embryos. The anterior cell produced an embryo which lacked the post-trochal region; in the embryo formed from the posterior cell there was present a nearly typical post-trochal region. Delage (1899) had previously described a dwarf embryo from an egg-fragment of the same species. That annelid eggs, generally, are of the mosaic type is shown by the experiments of Penners (1924, 1926) with *Tubifex*, of Tyler (1930) with *Chætopterus*, and of Hatt (1932) with *Sabellaria*.

Wilson (1929) summarizes the evidence which indicates that there is no fundamental distinction between the mosaic and regulative types of ova. Among regulative eggs, where correlative differentiation, or embryonic induction, is most prominent, mosaic features can be found, and in mosaic eggs, there are suggestions that embryonic induction may play a part in early development. Wilson suggests that the polar lobe of such eggs as *Dentalium* may function as an organizing region similar to the dorsal blastoporal lip of amphibia, since only when the lobe is present does the larva develop the apical tuft and the post-trochal region. However, in the absence of transplantation experiments, no final conclusions could be reached.

Schleip (1929) describes a "natural experiment" in which a second polar lobe is added to the egg of *Dentalium*. Among giant eggs, some are found which appear to be fusions of two ova at their vegetal hemispheres. In these eggs, a single large polar lobe may be formed, which goes in its entirety into one of the cells. This leaves one egg with no lobe, and the other with two. However, such eggs do not develop. Schleip then tried to transplant isolated polar lobes to blastomeres, but all attempts were unsuccessful.

Transplantation experiments can readily be performed in the egg of *Sabellaria vulgaris*. In this species, large polar lobes, similar to those found in *Dentalium*, are formed in the course of the first three cleavages. Although the egg is only about sixty micra in diameter, it is not difficult to remove the polar lobe or to separate individual blastomeres. Both blastomeres and polar lobes can be fused together in desired combinations and the eggs reared through a well-developed trochophore stage. In all, 247 successful transplant operations have been studied, including 80 in which either the first or second polar lobe was transplanted.

Material and Method

All experiments reported in this paper were performed at the Marine Biological Laboratory, Woods Hole, Mass., during the summers of 1935, 1936, and 1937. The animals used were dredged from Vineyard Sound and the eggs were obtained in the manner described elsewhere by the writer (1937). The egg of *Sabellaria vulgaris* possesses a tough, wrinkled vitelline membrane which resists cutting with glass needles. In addition, within the membrane, in the perivitelline space, there is a dense jelly which makes difficult the separation of individual blastomeres and which completely prevents bringing blastomeres together. Thus, to perform transplantations, it is necessary to first remove both membrane and jelly from the egg.

The vitelline membrane is removed from fertilized eggs of *Sabellaria* by treatment with an isotonic solution of NaCl, brought to pH 9.6 by the addition of Na_2CO_3 (Novikoff, 1938). In most cases, the eggs are thus treated within the ten minutes that elapse between the formation of the first polar body and the second. After they have been washed once in sea water, the denuded eggs are placed into a Syracuse dish of freshly-filtered sea water. The dish is allowed to remain without disturbance on the stage of a dissecting microscope. Within two or three minutes, the eggs have settled and are adhering to the glass bottom of the dish. By means of fine glass needles, each egg is then lifted from the jelly which remains adherent to the bottom. This process may have to be repeated several times in order to remove the jelly completely. The jelly being invisible, its removal can best be ascertained by bringing together the individual eggs; they come into contact with each other only when the jelly has been removed. At this time the eggs are quite sticky and if allowed to adhere too long to the dish they flatten out. Since such flattened eggs do not develop normally, it is important that the eggs be lifted, at close intervals, from the bottom of the dish.

The eggs are cut, free-hand, under the dissecting microscope. In order to determine the orientations of cells when fused, small spots are marked on the eggs before they are cut. This is done by bringing into contact with the surface of the egg the open end of a fine capillary tube filled with agar, in which is dissolved a vital dye such as Nile Blue sulphate. In order to fill an exceedingly fine bore, the following procedure is followed. A short piece of capillary tubing is partly filled by immersing one end in a warm solution of the agar containing the dye. When the agar has cooled, that part of the tube which has no agar is heated by a microflame and pulled out to the desired width of bore. The other end of the tube is then sealed off and the microflame applied to the part of the agar nearer the narrowed end. The agar melts and moves into the free end of the tube. On cooling, the agar, in many cases, remains at the opening of the tube. The tube is brought into contact with the egg by means of a Zeiss- Peterfi micromanipulator. The length of time during which the agar must remain in contact with the egg varies with the concentration of dye used. In some cases, where it is possible to determine the polarity of the isolated cell without previously staining a particular region, the entire cell is stained before transplantation. This is usually done before the membrane is removed, and a dilute solution of Nile Blue sulphate in sea water is used.

To effect the fusion of blastomeres it is only necessary to bring them into contact with each other, after the membrane and jelly have been removed. It is usually sufficient to press them together for several seconds, although this may, in some cases, have to be repeated several times before they finally stay together.

Following the operation, the eggs generally develop into swimming larvæ within five to six hours after fertilization. They can at that time no longer be left in the open dish for they soon swim to the surface, where they are quickly torn by the surface film. The procedure which leaves the least amount of surface exposed to the air and which, at the same time, is most convenient for the detailed study of the living larvæ, is to allow the embryos to develop in small drops of sea water between a glass slide and coverslip. When an embryo begins to show signs of movement, it is transferred, by means of a mouth pipette (Hörstadius, 1937*a*), to a small drop of sea water. Evaporation from the drop is prevented by sealing the edges of the coverslip with a thin layer of vaseline.

The results to be described are based on a total of 403 experimental larvæ, 156 developing from isolated blastomeres and 247 from fusions of various blastomeres. The percentages of larvæ surviving are: 73

per cent on the first day, and 51 per cent on the second day, from isolated cells; 91 per cent on the first day, and 74 per cent on the second day, from combinations of blastomeres. As described in the first paper of the present series (Novikoff, 1938), a great number of the larvæ arising from denuded eggs develop into exogastrulæ, in which there is no internal gut and the endoderm cells are turned inside out. Under the coverslips, all structures differentiate in the larvæ, just as they would in larger volumes of sea water, with the exception of the orange-red eye spot, and, to some extent, the yellow-green chromatophores. These are frequently absent or irregular. That the failure of the eye spot and the chromatophores to develop normally is due to some general factor such as lowered oxygen tension or increased pressure, is indicated by the development of normal eggs (i.e., eggs from which the membranes have not been removed) under similar conditions. Although the cell arrangement and the tissues of the larvæ from such eggs are manifestly similar to those of normal larvæ, the eye spot does not form and the chromatophores show the same variable character as do those of the membraneless larvæ.

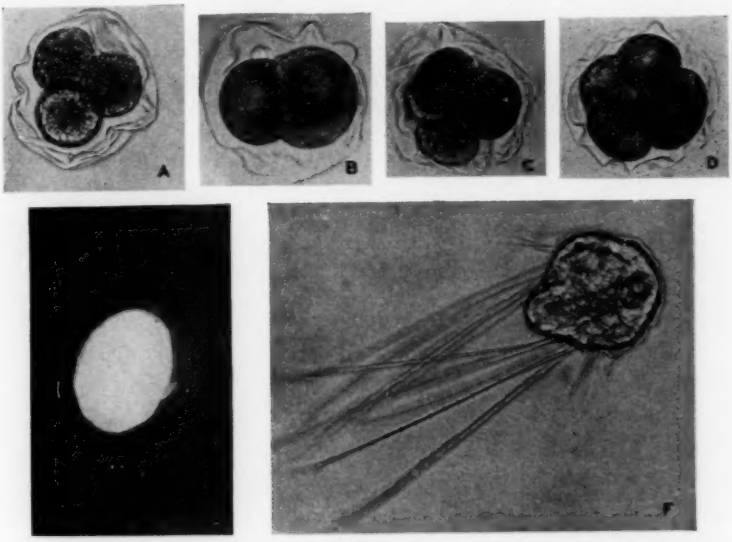
Normal Development

Changes in the egg of *Sabellaria vulgaris* at maturation and during fertilization are described elsewhere (Novikoff, in press). Both polar bodies are formed after insemination and cleavage begins approximately twenty minutes [1] after the extrusion of the second polar body.

In the course of the first cleavage division, a large part of the vegetal hemisphere of the egg becomes constricted in the form of a spherical lobe. Figure 1, *A* is a photograph of an egg at the "trefoil" stage, when the polar lobe is at its maximum size. When viewed from the side, the lobe appears to be equal in size to either of the first two blastomeres. But when viewed from the vegetal pole, it is seen to be considerably smaller. The visible constituents of the lobe cytoplasm do not differ from that of either blastomere, except that there is no spindle area in the lobe. About fifteen minutes after it first appears, the polar lobe flows into one of the blastomeres. This blastomere is the *CD* cell and it is now much larger than the other, the *AB* blastomere (Fig. 1, *B*). Figure 1, *C* shows an egg during the second cleavage, when the second polar lobe is at its maximum size. This lobe forms in the *CD* blastomere only, and is smaller than the first lobe. When it flows back into one of the daughter cells at the completion of the division, the four quarter-blastomeres consist of two equal-sized cells, *A* and *B* (the products of the division of *AB*), a slightly larger cell, *C*,

[1] All time intervals are for room temperatures, varying from 19° to 25° C.

and a much larger cell, *D* (Fig. 1, *D*). It is the *D* blastomere which has received the contents of the second polar lobe. During the next division, when the micromeres are produced, a third polar lobe, formed



FIGS. 1–10. All figures, except 1 and 5, are composed of camera lucida drawings of living larvæ, magnified approximately 260 times. Abbreviations used are:

p	prototroch
at	apical tuft
ac	apical cilia
ec	cilia of exogastrulated endoderm cells
pc	posterior cilium
dc	dorsal cilium
pb	post-trochal bristle
e	eye
m	mouth

FIG. 1. Photomicrographs of living eggs of *Sabellaria vulgaris*. *A*. Trefoil stage. Side view. The polar lobe is slightly out of focus. *B*. Two-cell stage. Blastomere *CD* is to the right of *AB*. *C*. Second cleavage. The second polar lobe and two of the quarter-blastomeres are in sharp focus; the other two cells are not in focus. *D*. Four-cell stage. Seen from the vegetal pole. The large *D* cell is to the right and the *A* cell is uppermost. *E*. Early trochophore larva. Sixteen hours after fertilization. Photographed with dark-field illumination; shows apical tuft and prototrochal cilia. *F*. Later trochophore larva. Forty-eight hours after fertilization; shows stomach, intestine, dark eye spot, prototrochal cilia, and post-trochal bristles.

from the *D* cell, flows into the *D* macromere, 1*D*. This lobe is smaller than the second lobe and is more variable than the preceding lobes;

in many cases this lobe does not become distinctly separated from the dividing *D* cell. The later cleavages of the egg have not been described.

A detailed description of the development of the larva is presented in the first paper of this series (Novikoff, 1938). In the normal course of development, the ectoderm gives rise to the following structures: a prototroch of long active cilia; chromatophores; an apical tuft; non-motile apical cilia; paired chætæ-sacs from which extend long, serrated bristles; a single posterior non-motile cilium; several long non-motile cilia on the dorsal surface; an eye spot; and a neurotroch of rapidly moving cilia. The endoderm differentiates into a tripartite gut consisting of œsophagus, stomach, and intestine. In many of the membraneless larvæ, the gut is exogastrulate and the three portions can not be distinguished. Of the ectodermal structures, those most easily observed in living embryos—and therefore the ones best suited for

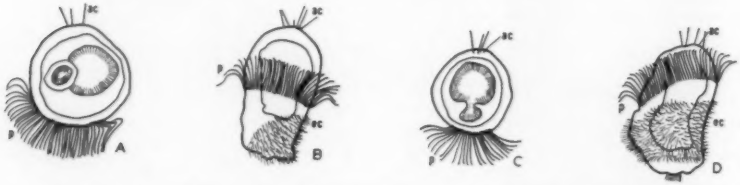


FIG. 2. Larvæ from isolated blastomeres. *A*. *E-PL1* larva, seventy-one hours; with internal gut. *B*. *E-PL1* larva, fifty hours; with exogastrulated endoderm. *C*. *AB* larva, fifty-one hours; with internal gut. *D*. *AB* larva, fifty-six hours; with exogastrulated endoderm.

the present study—are the apical tuft, the post-trochal bristles, the prototrochal cilia, and the apical cilia. The apical tuft forms at about six hours after fertilization, and persists for approximately twenty hours (Fig. 1, *E*). Before it disappears, there develop at the apical end, a number of stiff cilia; these apical cilia remain throughout larval development. The prototrochal cilia appear at about the same time as the apical tuft and they remain throughout larval development. The post-trochal bristles make their appearance toward the end of the first day of development. They increase in length and number as development progresses (Fig. 1, *F*).

ISOLATION EXPERIMENTS

E-PL1.—An egg from which the polar lobe is removed at the trefoil stage is labelled *E-PL1*. The first cleavage of *E-PL1* differs from the normal second cleavage in that no polar lobe is formed. As a result,

the quarter-blastomeres are equal in size. In addition, no lobe is formed at the next division. The larvæ of such eggs differ from the normal larva in that: (1) no apical tuft forms; (2) no post-trochal region (including the bristles) appears; and (3) the prototrochal cilia, although of the same size as in normal larvæ, are at the posterior end of the larval ectoderm (Fig. 2*A*, *B*). Although no apical tuft is present, at approximately twenty-four hours after fertilization the typical non-motile cilia appear.

AB.—The isolated *AB* cleaves without the formation of polar lobes and gives rise to a spherical larva similar to that of *E-PL1*. It lacks the apical tuft and post-trochal bristles. It develops normal prototrochal and apical cilia, although the former are situated at the posterior end of the larval ectoderm (Fig. 2*C*, *D*).

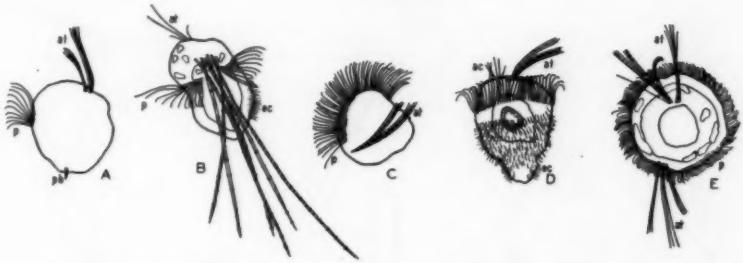


FIG. 3. Larvæ from isolated blastomeres. *A*. *CD* larva, twenty-five hours. *B*. *CD* larva, eighty-five hours. *C*. *CD-PL2* larva, fifty-six hours. *D*. *E-PL2* larva, forty-five hours. (On the next day, the apical tuft was no longer present.) *E*. *E-PL2* larva, eighteen hours. It is exceptional in that it possesses two apical tufts.

CD.—During the first cleavage of *CD* there is formed a polar lobe of the same size as the normal second lobe. After the division, it flows into the *D* cell. At the second cleavage, another, smaller lobe forms from the *D* cell. This lobe has the variable character of the normal third lobe. The early *CD* larvæ appear to be quite normal—the prototrochal cilia are in their usual position and the typical apical tufts are formed (Fig. 3, *A*). However, since the cilia and tuft are of the normal size, they are, proportionately, too large for these reduced larvæ. Later, the paired bristles are formed. However, no apical cilia develop, and in many instances (17 out of 29), the apical tuft does not disappear when it does in controls (Fig. 3, *B*).

CD-PL2.—If during the course of the first cleavage of the *CD* blastomere, the polar lobe is removed, the next cleavage occurs without the formation of a lobe. The *CD-PL2* larva possesses a typical apical

tuft but lacks the post-trochal structures as well as the apical cilia (Fig. 3, *C*).

E-PL2.—It is with difficulty that the second polar lobe is removed from a whole egg. In each of the three larvæ obtained, normal apical tufts and prototrochal cilia develop. (One larva possesses *two* apical tufts—Fig. 3, *E*.) The older larvæ show no post-trochal structures, but do develop apical cilia (Fig. 3, *D*).

BC.—Before the cells have shifted their position after the second cleavage, it is possible to divide them so that *BC* is separated from *AD*. The *BC* combination forms no polar lobe and gives rise to a larva which possesses the typical apical tuft and prototrochal cilia, and a few apical cilia. It lacks completely the post-trochal region (Fig. 4, *B*).

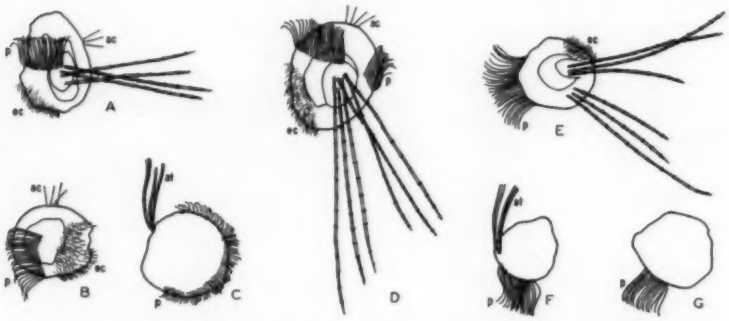


FIG. 4. Larvæ from isolated blastomeres. *A*. *AD* larva, forty-nine hours. *B*. *BC* larva, fifty hours. *C*. *ABC* larva, twenty-six hours. *D*. *ABD* larva, sixty-six hours. *E*. *D* larva, forty-seven hours. *F*. *C* larva, thirty-three hours. *G*. *D-PL3* larva, forty-one hours.

AD.—At the first cleavage of *AD*, a small lobe, which has the variable character of the normal third lobe, forms in the *D* cell. The larva developing from this combination of cells forms no apical tuft. It possesses normal prototrochal cilia and the typical post-trochal region from which extend the bristles. Apical cilia are also present (Fig. 4, *A*).

ABC.—By destroying the *D* cell at the completion of the second cleavage, *ABC* combinations are obtained. No polar lobe is formed at the first cleavage. The larva which develops possesses the typical apical tuft and apical cilia. However, no post-trochal bristles are produced (Fig. 4, *C*).

ABD.—Destruction of the *C* cell leaves the *ABD* blastomeres. At the next cleavage, the *D* cell forms a small polar lobe. The *ABD*

larvæ develop normal prototrochal cilia, but no apical tufts. Later in development, apical cilia and post-trochal bristles appear (Fig. 4, *D*).

C.—No polar lobes are formed during the cleavage of the *C* blastomere. The larva developing from the *C* cell develops typical prototrochal cilia and apical tuft. But neither post-trochal structures nor apical cilia are formed (Fig. 4, *F*).

D.—During the first cleavage of the *D* cell, the small variable lobe appears and passes into the macromere, 1*D*. The *D* larva has typical prototrochal cilia and post-trochal bristles. But it develops no apical tuft and no apical cilia (Fig. 4, *E*).

D-PL3.—In some cases, the lobe formed by the *D* cell constricts sufficiently so that it may be removed. The larva developing from

TABLE I

Summary of larvæ obtained from isolation experiments.

	Number Operated	Number Surviving	Apical Tufts +	−	?	Post-trochal Bristles +	−	Prototrochal Cilia +	−	?	Apical Cilia +	−	?
E-PL1	30	21	0	21		0	11	21	0		14	0	
AB	36	25	0	24		0	18	23	0	1	11	6	1
CD	56	47	37	1	9	32	1	42	0	5	1	26	6
CD-PL2	8	5	5	0		0	4	5	0		0	3	1
E-PL2	3	3	3	0		0	2	3	0		2	0	
BC	10	8	6	2		1	5	8	0		6	0	
AD	6	3	0	3		3	0	3	0		2	0	1
ABC	10	8	7	0	1	0	5	8	0		4	1	
ABD	7	5	0	5		4	0	5	0		4	0	
C	17	11	7	3	1	0	7	11	0		0	7	
D	23	15	0	12	3	9	4	15	0		0	9	4
D-PL3	7	5	0	5		0	3	5	0		0	3	
	213	156											

the *D-PL3* lacks the post-trochal structures as well as the apical tuft and apical cilia (Fig. 4, *G*).

Table I summarizes the isolation experiments. All combinations of cells develop prototrochal cilia. But only those combinations which include the substance of the *C* cell (earlier found in the first polar lobe) develop apical tufts; only those which have the 1*D* cell materials (and previously found in the three polar lobes) form the post-trochal region; and the apical cilia develop only when the *A* or *B* cell is present. Three exceptions, a *BC* larva with bristles, a *CD* larva with apical cilia, and an *E-PL2* larva with two apical tufts, will be discussed later.

Behavior of Isolated Polar Lobes

The changes in form of isolated polar lobes have been described in *Dentalium*, by Wilson (1904*b*) and in *Ilyanassa*, by Morgan (1933, 1935). In *Dentalium*, the isolated lobe usually constricts periodically to form lobe-like structures by a process which simulates the formation of polar lobes in the whole egg. There are three such constrictions, and they occur at approximately the same time as do the cleavages of the whole egg. One case is described in which the final constriction gives rise, not to a temporary lobe, but to a distinctly separated portion of cytoplasm. Wilson interprets the first of these changes as the formation of lobes within the isolated polar lobes, at the time when the whole egg normally forms lobes. The final stage he regards as a

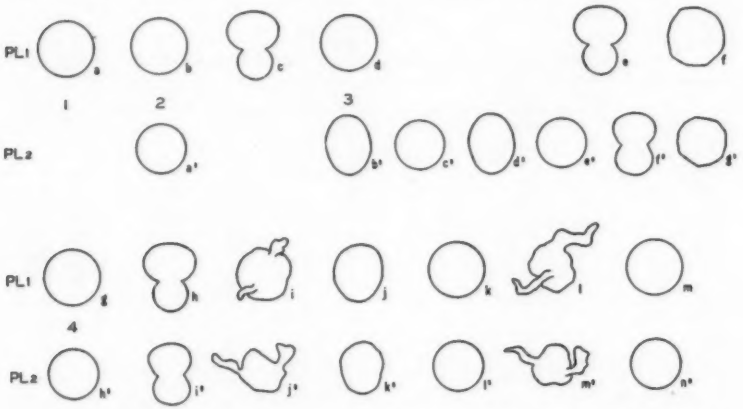


FIG. 5. Form changes in isolated polar lobes. *PL1*, first polar lobe. *PL2*, second polar lobe. Numerals 1–4 indicate the time of the first four cleavages of the whole egg. Explanation in text.

permanent division of the isolated lobe into two at the time of the fourth cleavage of the whole egg, in which the material of the polar lobe no longer forms a temporary polar lobe, but is permanently cut off by a cell division. Morgan argues against a literal interpretation of the form changes in the isolated lobe as lobes. In *Ilyanassa*, he finds that: (1) The changes in the lobe are not strictly synchronous with the cleavage of the whole egg. (2) The constrictions in isolated lobes come and go at least three times, whereas in the whole egg, the lobe would appear only once more. (3) The change in shape does not, strictly speaking, give a reduced picture of the changes in the whole egg; and, (4) the form changes in the lobe resemble more the process of

micromere constriction rather than lobe formation. Therefore, Morgan interprets the later changes of the isolated lobe as related to the constriction of micromeres, and not to the formation of polar lobes.

First Polar Lobe.—Isolated first polar lobes of *Sabellaria* were observed continuously for two to three hours. Although, as both Wilson and Morgan found, the behavior is somewhat variable, most lobes show a remarkable constancy in their changes. Of the 31 isolated lobes studied, 25 were of the type drawn in Fig. 5; the other six were more or less variable.

No change in the shape of the isolated lobe occurs at the time of the first cleavage of *E-PL1*, i.e., at the time of the second cleavage of the whole egg (Fig. 5, *b*). About ten to fifteen minutes after the first cleavage of *E-PL1*, the lobe is deeply constricted to form a definite lobe-like structure (Fig. 5, *c*). At the time of the second cleavage, the lobe is spherical (Fig. 5, *d*), but another deep constriction appears about ten to fifteen minutes later (Fig. 5, *e*). As this second constriction disappears, a slight flattening of the lobe occurs (Fig. 5, *f*). This lasts for a short time, and when *E-PL1* cleaves for the third time, the lobe is again spherical (Fig. 5, *g*). A third constriction forms after the third cleavage of *E-PL1* (Fig. 5, *h*), but before it disappears completely, the entire cell becomes irregular in outline, and long irregular pseudopodia are formed (Fig. 5, *i*). These are later withdrawn (Fig. 5, *j*) and the cell again becomes spherical (Fig. 5, *k*). However, it remains in this condition for only a short time, until the irregular pseudopodia are formed again (Fig. 5, *l*). The extension and retraction of the pseudopodia is not synchronous with cleavage, and continues until the lobe cytolyzes. In several cases, the process was still going on twenty-eight hours after the removal of the lobe; in one case, it was observed up to forty-eight hours after the separation of the lobe.

Second Polar Lobe.—The second polar lobes were removed from five isolated *CD* cells, and their behavior followed continuously for two hours. Four of the five lobes produced fairly deep constrictions twice (Fig. 5). The other produced only the first of the two constrictions; at the time when the second constriction would form, the cell elongated somewhat without constricting. The behavior of the four lobes which formed the two constrictions was fairly uniform. When *CD-PL2* divides for the first time, i.e., at the time of the third cleavage of the whole egg, the isolated lobe elongates slightly (Fig. 5, *b'*) and within a minute is rounded out (Fig. 5, *c'*). A similar elongation forms again, in three of the lobes, in about eight to ten minutes after the first (Fig. 5, *d'*). At about seventeen to eighteen minutes after the first cleavage, the lobe develops a constriction which persists for two

minutes (Fig. 5, *f'*). As the constriction disappears, the lobe flattens slightly (Fig. 5, *g'*). Within a few minutes, the second cleavage of *CD-PL2* occurs. Following the second cleavage a second constriction forms (Fig. 5, *i'*), and as it disappears the cell becomes irregular and gives rise to pseudopodia (Fig. 5, *j'*). The behavior of the pseudopodia is similar to those of isolated first lobes (Fig. 5, *j'–n'*).

The constrictions formed by the isolated first and second polar lobes resemble in appearance the polar lobes formed by the dividing ovum. Although these constrictions do not occur at the same time as the cleavages of the ovum, they must, in some way, be related to events taking place in the egg during division. This is brought out by a comparison of the time elapsing between successive cleavages of the lobeless egg and the constrictions of the isolated lobe, in the twenty-five cases where the first polar lobe was removed and the five in which the second lobe was removed. The average time between the first and second cleavages of *E-PL1* is 20.6 minutes and that between the first and second constrictions of the isolated first lobe 18.6 minutes. The time between the second and third cleavage of *E-PL1* is 24.6 minutes and that between the second and third constriction is 21.2 minutes. For the isolated second lobes, the average time between the two constrictions is 21.8 minutes, while the interval between the first and second division of *CD-PL2* is 21.0 minutes. The changes in the form of the isolated lobes are apparently synchronous with the cleavages of the ovum, except that all events in the isolated lobes are pushed back by a delay in the appearance of the initial constriction. This delay is approximately ten to fifteen minutes for the isolated first lobe and about seventeen to eighteen minutes for the isolated second lobe.

On the basis that the periodic constrictions formed by isolated polar lobes are correlated with cytoplasmic changes occurring in whole eggs at the time of the cleavages in which polar lobes are formed, we would expect the isolated second lobe to form one fewer constriction than the isolated first lobe. The isolated first lobe forms three constrictions; the isolated second lobe two. And, as would be expected, the time elapsing between the first and second constrictions of the isolated second lobe (21.8 minutes) is almost identical with the time between the second and third constrictions of the isolated first lobe (21.2 minutes). However, certain differences between the behavior of the isolated lobes and that of polar lobes of the whole egg must be noted. The whole egg forms two polar lobes after the formation of the first lobe, and one after the second. The isolated first lobe, however, forms three constrictions and the isolated second lobe two.

Although there is a decrease in size of successive lobes in the whole egg, the constrictions of the isolated lobe are all of approximately the same size. But the facts to be emphasized are: (1) that there reside in the isolated polar lobes materials which take part, independently of the nucleus or the mitotic apparatus, in reactions affecting the tension at the surface of the cell, and (2) that these reactions in isolated lobes occur synchronously (if we discount the initial delay) with events in the whole egg, or in the egg from which the polar lobe has been removed.

TABLE II

Differentiation of larvæ after transplantation of polar lobes and blastomeres.

Type of Operation	Number Operated	Number Survived	Larvæ with:			Larvæ with:			Larvæ with:		
			0 Apical Tuft	1 Apical Tuft	2 Apical Tufts	0 Sets of Bristles	1 Set of Bristles	2 Sets of Bristles	0 Groups of Apical Cilia	1 Group of Apical Cilia	2 Groups of Apical Cilia
A. Polar lobe transplants											
Transplant *Host*											
PL1 Whole egg	26	23	7	16	0	8	14	0	7	15	0
AB	35	32	32	0	0	20	0	0	5	14	0
E-PL1	15	14	14	0	0	10	0	0	3	7	0
PL2 Whole egg	6	5	0	5	0	2	2	0	1	3	0
AB	6	6	6	0	0	3	0	0	0	3	0
B. Transplantation of blastomeres to whole egg											
Transplant											
CD	9	9	0	2	7	0	1	6	1	5	0
C	10	10	0	4	6	0	8	0	2	6	0
D	16	14	2	10	0	0	4	10	2	12	0
AB	11	11	2	9	0	2	8	0	1	4	5
C. Transplantation of blastomeres to *E-PL1*											
Transplant											
CD	16	16	2	11	0	2	12	0	5	9	0
C	20	18	6	12	0	14	0	0	5	8	0
D	15	15	15	0	0	0	13	0	4	9	0
CD-PL2	4	3	1	2	0	2	0	0	0	2	0
AB	12	11	11	0	0	9	0	0	0	3	6
Whole egg	3	3	1	2	0	0	3	0	0	1	2
D. Fusions of blastomeres											
Combination											
CD, CD	20	19	0	11	8	2	2	11	15	0	0
CD, D	9	8	0	7	0	1	1	5	7	0	0
CD, C	6	4	1	2	1	0	2	0	2	0	0
C, C	4	3	0	2	0	2	0	0	2	0	0
AB, AB	12	10	10	0	0	8	0	0	1	2	5
3 *AB*'s	3	2	2	0	0	1	0	0	0	0	1
5 *AB*'s	1	1	1	0	0	1	0	0	Three groups of cilia		

TRANSPLANTATION EXPERIMENTS

Transplantation of Polar Lobes

Transplants of first and second polar lobes were made to whole eggs at the trefoil, two-cell, and four-cell stages, to *AB* blastomeres, and to *E-PL1*. The lobes were placed at the animal pole, at the vegetal pole, and at the equator of the dividing egg. Although

fused to the blastomeres, the lobes go through essentially the same form changes as do isolated lobes. When cleavage progresses, the blastomeres may grow over and completely enclose the lobe, or the lobe may remain at one end of the larva. The lobe is still part of the larva when the cilia appear (Fig. 6*A*, *C*). Prototrochal cilia form in all larvæ, and the apical tufts develop only in those which include *C* cells (Table II, *A* and Fig. 6). At about fifteen hours after fertilization, the lobe, more or less completely cytolyzed, is extruded from the larva (Fig. 6, *D*). This is generally followed by a cytolysis of a portion of the embryo, especially in those cases where the lobe was deeply embedded within the embryo. Thus, only 59 out of the 80 larvæ

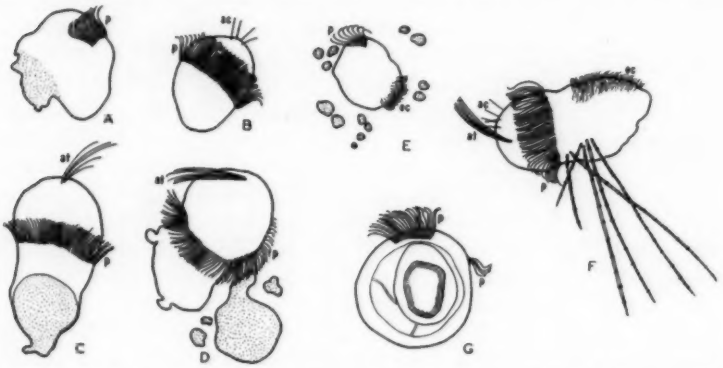


FIG. 6. Differentiation of larvæ after transplantation of polar lobes. *A*. *AB* plus *PL1* larva, 11½ hours. The lobe (stippled) is within the larva. *B*. *AB* plus *PL1* larva, 24 hours. The larva has moved away from the cytolyzed spheres of the ejected lobe. *C*. *E* plus *PL1* larva, 13 hours. The lobe (stippled) is within the larva. *D*. *E* plus *PL1* larva, eighteen hours. The lobe has been ejected. *E*. *AB* plus *PL1* larva, 45 hours. The ejected lobe has broken up into small spheres. *F*. *E* plus *PL1* larva, 26 hours. *G*. *E-PL1* larva, 50 hours. The first polar lobe was in contact with the dividing egg for five hours, at the end of which time it was removed.

survived beyond twenty-four hours. Where the larva is not greatly damaged by the loss of the lobe, differentiation progresses normally. The *AB* and *E-PL1* larvæ form apical cilia but no post-trochal regions (Fig. 6, *B*). The larvæ produced by entire eggs lose their apical tufts when apical cilia appear, and they develop post-trochal regions with typical bristles (Fig. 6, *F*).

In many cases, the polar lobe was stained heavily with Nile Blue sulphate, before being transplanted. The dye diffused from the lobe into the adjacent cells, so that the larval tissues acquired a pronounced blue coloration.

To avoid possible effects of the lobe cytolysis on the differentiation of the larva, the lobe was, in fifteen cases, allowed to remain fused to the dividing cells only until the embryo began to show signs of movement, when it was removed. The contact is long enough so that the blue color of the dye diffuses from the lobe into adjacent cells. The larvæ which develop from such cells are in no essential way altered by contact with the lobe (Fig. 6, *G*).

The presence of the polar lobe, fused to the larva for five hours (when it is removed), or within the larva for eleven hours (at which time it is ejected), does not induce the formation of larval structures.

Transplantation of Blastomeres to Whole Eggs

Table II, *B* summarizes the types of transplants made to whole eggs, at the trefoil, two-cell, and four-cell stages. The transplants

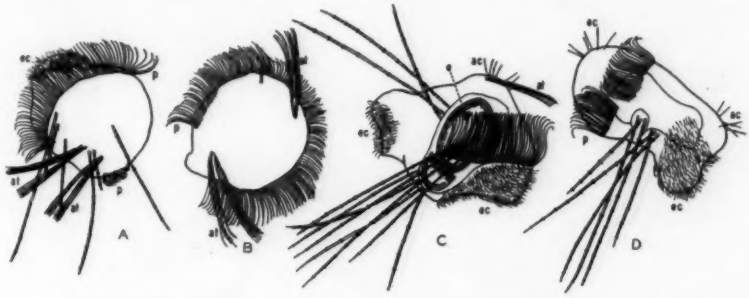


FIG. 7. Differentiation of larvæ after transplantation of blastomeres to whole eggs. *A*. *E* plus *CD* larva, 27 hours. *B*. *E* plus *C* larva, 24 hours. *C*. *E* plus *D* larva, 79 hours. *D*. *E* plus *AB* larva, 40 hours.

are in some cases stained with Nile Blue sulphate before being fused to the host. It is possible to follow the stained region through the first day of development, but beyond this time the dye is not visible. The orientation is varied, but no correlation between any particular orientation and type of development is found.

The differentiation of the transplanted *AB*, *CD*, *C*, or *D* blastomeres does not bring about the development of additional structures in the host; those structures which are duplicated in the larva arise from the self-differentiation of the transplanted cells. Seven of the nine *E* plus *CD* larvæ show two apical tufts; six (out of the seven surviving) show two sets of post-trochal bristles (Fig. 7, *A*). In six of the ten *E* plus *C* larvæ, two apical tufts are visible (Fig. 7, *B*). Ten of the fourteen *E* plus *D* larvæ possess two sets of bristles (Fig. 7, *C*). The *E* plus *AB* larvæ show no duplication of either the apical tuft or

bristles, but five of the ten possess two distinct groups of apical cilia (Fig. 7, *D*).

Transplantation of Blastomeres to E-PL1

The polar lobes are removed from eggs at the trefoil stage, and in their place are put half- or quarter-blastomeres, or whole eggs. In other experiments, transplants are placed at the animal pole, or to the side of *E-PL1*. As in previous operations, the transplanted cells are first stained, either *in toto*, or locally, to mark their polarity. The resultant larvæ are summarized in Table II, *C* and a few of the various kinds are shown in Fig. 8. In all larvæ, the host cells give rise to

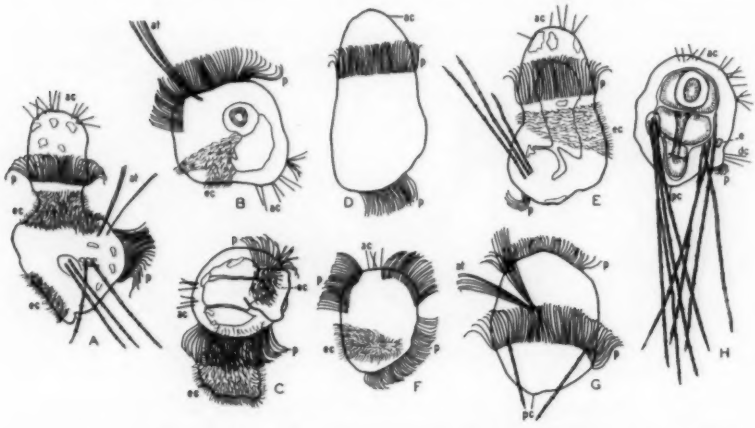


FIG. 8. Differentiation of larvæ after transplantation of blastomeres to *E-PL1*. *A*. (*E-PL1*) plus *CD* larva, 43 hours. *B*. (*E-PL1*) plus *C* larva, 30 hours. *C*. (*E-PL1*) plus *C* larva, 48 hours. *D*. (*E-PL1*) plus *D* larva, 17 hours. *E*. Same larva as shown in *D*, 43 hours. *F*. (*E-PL1*) plus *AB* larva, 54 hours. *G*. (*E-PL1*) plus *E* larva, 25 hours. *H*. Same larva as shown in *G*, 91 hours.

prototrochal cilia, and in at least 36 of 55, they form apical cilia. They form no apical tuft and no post-trochal bristles. The apical tufts, post-trochal bristles, or supernumerary prototrochal and apical cilia which are present in the larvæ arise only through the self-differentiation of the transplant.

Fusions of CD, C, and D Blastomeres

Thirty-four operations involving combinations of *CD* cells with *CD*, *D*, and *C* blastomeres, and fusions of two *C* cells were performed (Table II, *D* and Fig. 9). Among the 19 larvæ with the *CD*, *CD* combination, 8 show two apical tufts; in 11, only one tuft is distinctly

visible. Of the 14 larvæ surviving beyond the first day, 2 fail to develop post-trochal bristles, 2 form one set of bristles, and 11 develop two distinct sets. There are 7 larvæ with the *CD*, *D* combination. Each of these larvæ produces one apical tuft, and 5 of the 7 show two sets of post-trochal bristles. One larva forms one set of bristles and another forms none. Four larvæ of the *CD*, *C* constitution show the following: two apical tufts, 1; one apical tuft, 2; no apical tuft, 1. Two survive long enough to develop bristles; in each there is one set. Only two *C* plus *C* larvæ survive; each possesses only one apical tuft and none forms post-trochal bristles except those which include *D* cells.

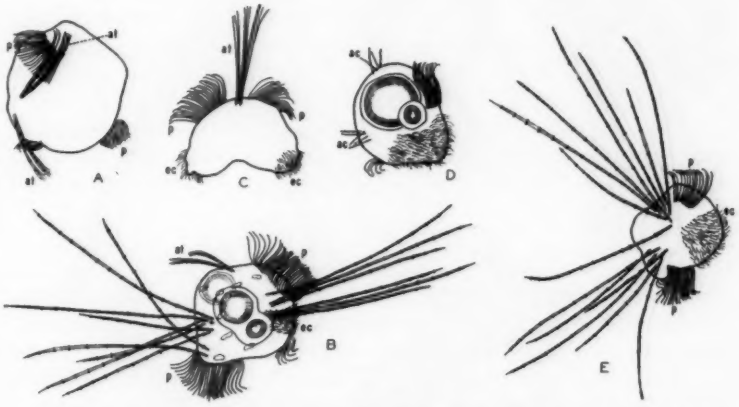


FIG. 9. Differentiation of larvæ arising from fusions of blastomeres. *A*. *CD* plus *CD* larva, 13 hours. *B*. Same larva as shown in *A*, 59 hours. *C*. *C* plus *C* larva, 28 hours. *E*. *CD* plus *D* larva, 55 hours. *D*. *AB* plus *AB* larva, 50 hours.

Fusions of AB Blastomeres

At the time of the first cleavage of isolated *AB* cells, various combinations are effected. Of the larvæ surviving, ten come from fusions of two *AB* cells, two from three *AB*'s, and one from five. Five of seven of the *AB*, *AB* larvæ show two sets of apical cilia, and two show one set; in the single surviving larva from the combination of three *AB*'s, two sets of apical cilia are seen; in the larva from the fusion of the five *AB*'s three distinct sets of apical cilia are visible. Although there is a superabundance of cellular material, none of the larvæ develops an apical tuft and none develops a post-trochal bristle (Table II, *D* and Fig. 9, *D*).

Fusions of Whole Eggs

Two eggs are fused at the trefoil stage or at the two-cell stage. The orientation of the two eggs with respect to each other is varied as follows: (1) the animal-vegetal axes of the ova remain parallel, but the eggs are rotated to different degrees; (2) one of the two eggs is inverted so that the fusion occurs at the animal poles or at the vegetal poles of the two eggs, with the rotation of the eggs varied as in (1).

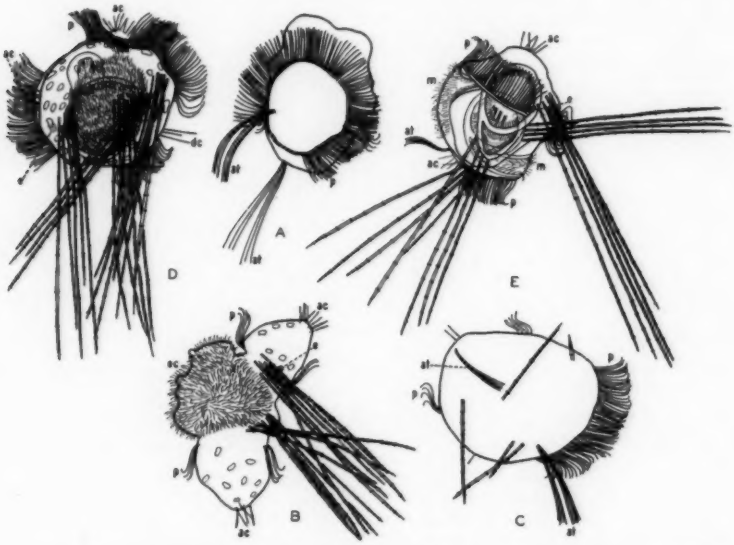


FIG. 10. Differentiation of larvæ arising from fusions of two eggs. *A*. Larva, 23 hours. *B*. Same larva as shown in *A*, 52 hours. *C*. Another larva, 27 hours. *D*. Another larva, 49 hours, with one internal gut. On the outer surface is an area of ciliated cells, devoid of chromatophores. *E*. Another larva, 55 hours, with two internal guts.

Of the twenty-five fusions effected, 23 larvæ are alive at the end of twenty hours of development, and 19 at the end of thirty-five hours. Of the 23, 2 larvæ show no apical tuft, 7 show one tuft, and 11 show two tufts. Among the 19 larvæ, the post-trochal bristles fail to form in one case, 4 larvæ have one set of bristles, and 14 have two sets; the apical cilia are not visible in 2 larvæ, 5 show one group of cilia, and 12 show two distinct groups. In none of the larvæ are there more than two apical tufts, or more than two sets of post-trochal bristles, or more

than two groups of apical cilia. Figure 10 includes three advanced larvæ of different types. In one type (Fig. 10, *B*), the endoderm cells of both eggs have been exogastrulated. In another (Fig. 10, *E*), there are two distinct internal guts, of fairly normal structure. In a third type (Fig. 10, *D*), only one internal gut is found. This gut has the typical tripartite character and is no larger than the normal gut. On the outer surface of the larva, a wide, delimited, area is devoid of the chromatophores characteristic of the ectoderm, and the cells of this area are covered with the rapidly-moving cilia, characteristic of the gut cells. The constitution of this area is therefore interpreted as the endoderm cells of one of the eggs. Instead of giving rise to a gut, these cells have become part of the outer covering of the larva; but in spite of their new location, they continue to differentiate as they would normally.

In two instances, three eggs are fused at the two-cell stage. The resultant larvæ develop three sets of post-trochal bristles. In one case, four eggs are fused at the two-cell stage, and the larva develops four sets of bristles.

Discussion of Results

By means of isolation experiments, the independent developmental capacities of early blastomeres of *Sabellaria vulgaris* were determined. These experiments included the usual separation of half- and quarter-blastomeres, and, in addition, the separation of other combinations of cells (*AD*, *BC*, *ABD*, and *ABC*). Also, the effect of the removal of three polar lobes was studied. Table I classifies the 156 surviving larvæ, with respect to the presence or absence of the prototroch, apical tuft, post-trochal bristles, and apical cilia. Prototrochal cilia were present in all larvæ. The apical tuft formed only in those larvæ which included the first polar lobe and the *C* cell. The post-trochal bristles developed only when the three polar lobes and the 1*D* cell were present. The differentiation of apical cilia occurred only when either the *A* or the *B* cell was present. Two of the larvæ were exceptions to this conclusion: one *CD* larva, which developed apical cilia, and one *BC* larva, which developed post-trochal bristles. A third larva, of the *E-PL2* type, formed two apical tufts instead of one.

Having determined the fate of the blastomeres when isolated, the behavior of these same cells when placed in contact with each other atypically, or with isolated polar lobes, was investigated. The twenty-two types of transplantation experiments, including a total of 247 larvæ, are summarized in Table II. Not a single case is found in which the transplant had induced the formation of any particular structure

in the host. In any combination of blastomeres, apical tufts develop only when *C* cells are present, post-trochal bristles are dependent upon the presence of *D* cells, and apical cilia form only when either the *A* or *B* cell is included.

In Table III, the larvæ are classified on a different basis. The number of *C*, *D*, and *A* or *B* cells included in the makeup of the individuals are compared with the number of apical tufts, post-trochal structures, and apical cilia. In general, the number of apical tufts is

TABLE III

Classification of larvæ developing from transplantation experiments.

Number of *C* Cells	Total No. of Larvæ	Number of Larvæ with		
		0 Apical Tufts	1 Apical Tuft	2 Apical Tufts
0	88	88	0	0
1	95	21	74	0
2	64	3	28	33
Number of *D* Cells *	Total No. of Larvæ	Number of Larvæ with		
		0 Sets of Bristles	1 Set of Bristles	2 Sets of Bristles
0	69	69	0	0
1	63	14	49	0
2	62	4	12	46
Number of *A* (or *B*) Cells	Total No. of Larvæ	Number of Larvæ with		
		0 Apical Cilia	1 Group of Apical Cilia	2 Groups of Apical Cilia
0	26	26	0	0
1	128	35	93	0
2	49	4	15	30

* Two larvæ had 3 *D* cells; both had 3 sets of bristles. One larva had 4 *D* cells; it had 4 sets of bristles.

determined by the number of *C* cells present, the number of sets of post-trochal bristles by the number of *D* cells, and the number of groups of apical cilia by the number of *A* (or *B*) cells. The absence of a higher correlation, particularly in the cases of the apical tuft and apical cilia, may possibly be due to several reasons: (1) The larvæ are actively swimming and, especially in small, healthy individuals, such minute structures may be overlooked. (The post-trochal bristles are much more readily seen. The correlation is higher with this structure than

with either of the other two.) (2) Some of the operated individuals may have been injured and the failure to form a particular structure may be a manifestation of their reduced vitality. (3) Actively swimming larvæ may have some of their cells torn away by the surface at the edge of the drop of water. It is in many cases not possible to determine whether this has occurred. (4) The mechanical effects of the neighboring tissues may prevent the cells from giving rise to the particular structure. If true, this effect might possibly be conceived as a kind of regulatory process.

The most significant feature of the transplantation experiments is that in no instance is the number of apical tufts greater than the number of *C* cells, nor the number of post-trochal regions greater than the number of *D* cells, nor the number of groups of apical cilia greater than the number of *A* (or *B*) cells.

Of special interest are several larvæ which developed from a fusion of two eggs at the two-cell stage. In these larvæ (Fig. 10, *D*), there is present a single gut, of normal size and typical tripartite structure. In one of the two eggs, the cells whose prospective value is gut endoderm have been incorporated into the outer covering of the larva. Although "ectodermal" in the sense of location, these cells continue to differentiate into endoderm, i.e., they do not develop chromatophores and they become ciliated on their outer surface. The self-differentiation of the endoderm in this position indicates, as does the development of exogastrulæ (Novikoff, 1938), the complete independence of endodermal and ectodermal differentiation in *Sabellaria vulgaris*.

Since the polar lobe, as well as any of the quarter- or half-blastomeres, does not affect the differentiation of any cell through contact with that cell, it is not possible to consider the polar lobe an "organizer," in the sense of Spemann (cf. Wilson, 1929, pp. 202–205, and Huxley and deBeer, 1934, pp. 171–172). The experiments of Tyler (1930) have demonstrated that when the first cleavage of the *Chætopterus* egg is made equal—either through the retraction of the polar lobe into the smaller, *AB*, blastomere, or through the cleavage furrow dividing both the egg and the polar lobe equally—then the two half-blastomeres are totipotent. In both types of equal cleaving eggs, the *AB* blastomere receives polar lobe material. If allowed to develop *in toto*, such eggs produce double monsters of the cruciata type. (At the second cleavage, two very small polar lobes may be formed.) When separated, each half-blastomere produces a fairly normal larva. There is in reality no "*AB*" cell; each cell behaves like a *CD*. But there is no evidence that the substance, whose altered distribution

changes the prospective value of the cell from that of an *AB* to that of a *CD* cell, is located within the polar lobe. The fact that a double embryo is produced when the polar lobe goes in its entirety into the *AB*, i.e., that the *CD* can produce an embryo without the materials of the polar lobe, indicates the complexity of the situation. That the vegetal hemisphere of the molluscan or annelid egg possesses a particular substance at the time of the first cleavage is well established by the work on *Ilyanassa* (Crampton, 1896), on *Dentalium* (Wilson, 1904*b*), on *Tubifex* (Penners, 1924, 1926), and on *Sabellaria* (Hatt, 1932, and the present paper). In *Tubifex*, if the first cleavage of the whole egg or the *CD* blastomere is made to take place equally instead of unequally (through heat or lack of oxygen)—and the pole-plasms are distributed equally to the two cells—double cruciata monsters are produced (Penners, 1924). A single case was observed in *Sabellaria* in which the first two blastomeres were of equal size. When isolated, each of the blastomeres produced polar lobes at the next two cleavages, and each gave rise to a larva possessing an apical tuft. The fact that each cell forms polar lobes and that the two cells are of equal size indicates that each cell probably received materials from the first polar lobe. It would, then, appear that some substance present in the first polar lobe *does* have the ability to change the course of development of a cell, but that this substance does not act by contact with a cell; it must become a part of the cell. Normally, only the *CD* cell develops an apical tuft since the materials of the lobe flow only into that cell. The exceptional production of apical cilia by a *CD* cell and the appearance of post-trochal bristles in a *BC* combination, mentioned earlier, may be due to a deviation in either of the first two cleavage furrows, with a resultant unusual allocation of materials. The doubling of the apical tuft in the *E-PL2* larva may also be due to an unusual pattern, in which the materials giving rise to apical tufts are separated into different cells.

In the so-called regulative eggs, it is in many cases possible to alter the course of differentiation of a cell by transplanting the cell to a new position in the developing embryo. By varying the stage at which the operation is performed, the time of determination of a structure may be ascertained. Due to the scarcity of similar experiments on mosaic eggs, relatively little information is available concerning the effect of one part of an embryo on another during the course of development, or the existence of inducing, or organizing, regions in these eggs. Penners (1926, 1934) destroyed varying numbers of mesodermal and ectodermal teloblasts of *Tubifex*, at different stages, to test (1) the inter-dependence of ectoderm and mesoderm during development, and

(2) the inducing capacity of the teloblasts. He found that the ectoderm and mesoderm show complete independent differentiation, except for a slight influence of the development of one upon the form and upon the rate of development of the other. The fact that following the destruction of the teloblasts the embryos continue to develop normally indicates that the teloblasts are not organizing centers. Hörstadius (1937*b*) combined various quartets of blastomeres of the sixteen-cell stage in *Cerebratulus;* he found no effect of one layer upon the differentiation of the others. These results are in agreement with those of the present investigation, in which the polar lobe, half-, and quarter- blastomeres are shown to be ineffective in directing the development of *Sabellaria*.

However, in another egg which was thought to be mosaic, the egg of the ascidian, effects of one cell upon the development of the other have been reported. Tung (1934) found that some factor outside the brain is responsible for the formation of the sense organ, in *Ascidiella*. Also, Tung found indications that the adhesive organ is induced. A recent paper by Rose (1937) reports that, similarly, in the egg of *Styela*, the eye spot is induced by the gray macromeres.

Summary

1. Isolation experiments on the egg of *Sabellaria vulgaris* demonstrate that the formation of the apical tuft in partial larvæ is dependent upon the presence of the first polar lobe and the *C* cell; that the post-trochal region develops only when the three polar lobes and the 1*D* cell are present; and that apical cilia form only if the *A* or *B* cell is included.

2. Form changes in isolated first and second polar lobes are described. The early changes are synchronous with the cleavages of the ovum, except that all events in the isolated lobe are delayed.

3. The results of the following transplantation experiments are reported: (*a*) Transplantation of polar lobes. (*b*) Transplantation of blastomeres to the whole egg. (*c*) Transplantation of blastomeres to *E-PL1*. (*d*) Fusions of half- and quarter-blastomeres. (*e*) Fusions of two eggs. In all combinations, complete self-differentiation of individual blastomeres occurs. Apical tufts develop only when *C* cells are present, post-trochal bristles are dependent upon the presence of *D* cells, and apical cilia form only when either the *A* or *B* cell is included.

4. The results of this investigation are compared with those from experiments on other mosaic eggs.

It is with pleasure that the writer expresses his gratitude to Pro-

fessor L. G. Barth for his untiring assistance and constant encouragement throughout the course of this investigation, and to Professor E. B. Wilson for his inspiring interest in the work.

BIBLIOGRAPHY

CRAMPTON, HENRY E., 1896. Experimental studies on gasteropod development. *Arch. f. Entw.-mech.*, **3**: 1.

DELAGE, YVES, 1899. Études sur la mérogonie. *Arch. de Zool. expér. et gén.* (Ser. III), **7**: 383.

HATT, PIERRE, 1932. Essais experimentaux sur les localizations germinales dans l'oeuf d'un Annélide (Sabellaria alveolata L.). *Arch. d'Anat. Micros.*, **28**: 81.

HÖRSTADIUS, SVEN, 1937*a*. Microdissection, Free-hand Manipulations. In Handbook of Microscopical Technique, edited by C. E. McClung. Paul B. Hoeber, p. 43.

HÖRSTADIUS, SVEN, 1937*b*. Experiments on determination in the early development of Cerebratulus lacteus. *Biol. Bull.*, **73**: 317.

HUXLEY, JULIAN S., AND G. R. DEBEER, 1934. The Elements of Experimental Embryology. Cambridge. University Press.

MORGAN, THOMAS H., 1933. The formation of the antipolar lobe in Ilyanassa. *Jour. Exper. Zoöl.*, **64**: 433.

MORGAN, THOMAS H., 1935. The rhythmic changes in form of the isolated antipolar lobe of Ilyanassa. *Biol. Bull.*, **68**: 296.

NOVIKOFF, ALEX B., 1937. Sabellaria vulgaris. In Culture Methods for Invertebrate Animals, edited by Galtsoff et al. Comstock Publishing Co. Inc., p. 187.

NOVIKOFF, ALEX B., 1938. Embryonic determination in the annelid, Sabellaria vulgaris. I. *Biol. Bull.*, **74**: 198.

PENNERS, ANDREAS, 1924. Experimentelle Untersuchungen zum Determinationsproblem am Keim von Tubifex rivulorum Lam. I. *Arch. Mikr. Anat. u. Entw.-mech.*, **102**: 51.

PENNERS, ANDREAS, 1926. Experimentelle Untersuchungen zum Determinationsproblem am Keim von Tubifex rivulorum Lam. II. *Zeitschr. f. Wiss. Zool.*, **127**: 1.

PENNERS, ANDREAS, 1934. Experimentelle Untersuchungen zum Determinationsproblem am Keim von Tubifex rivulorum Lam. III. *Zeitschr. f. Wiss. Zool.*, **145**: 220.

ROSE, MERYL S., 1937. The induction of pigment spots in Styela partita (Abstract). *Anat. Rec.*, **70** (Supplement 1): 102.

SCHLEIP, WALDEMAR, 1929. Die Determination der Primitiventwicklung. Leipzig, Akad. Verlags.

TUNG, TI-CHOW, 1934. Recherches sur les potentialités des blastomères chez Ascidiella scabra. *Arch. d'Anat. Micros.*, **30**: 381.

TYLER, ALBERT, 1930. Experimental production of double embryos in annelids and mollusks. *Jour. Exper. Zoöl.*, **57**: 347.

WILSON, EDMUND B., 1904*a*. Mosaic development in the annelid egg. *Science*, **20**: 748.

WILSON, EDMUND B., 1904*b*. Experimental studies on germinal localization. I. The germ-regions in the egg of Dentalium. *Jour. Exper. Zoöl.*, **1**: 1.

WILSON, EDMUND B., 1929. The development of egg-fragments in annelids. *Arch. f. Entw.-mech.*, **117**: 179.

THE RELATION OF MORTALITY AFTER ENDOMIXIS TO THE PRIOR INTERENDOMICTIC INTERVAL IN PARAMECIUM AURELIA[1]

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Investigators of endomixis in *Paramecium* have frequently observed that animals often die during or soon after endomixis. According to Erdmann and Woodruff (1916), few *Paramecium caudatum* survived endomixis. In *Paramecium aurelia*, Caldwell (1933) found that death occurred 2.85 to 9.40 times more frequently at endomixis than in the middle of the period between endomixes. The present paper is a presentation of the results of a study of one of the factors determining such deaths, namely, the length of the preceding interendomictic interval. As will appear below, when the prior interendomictic interval is unusually long, endomixis results in a greater percentage of deaths than when the interval is of the ordinary duration. Moreover, the greater the interendomictic interval, the greater is the mortality resulting from endomixis, until, after very long intervals, endomixis invariably results in death.

In order to investigate this question, it was essential to have available, simultaneously, lines with normal interendomictic intervals and sister lines with unusually long intervals. This was accomplished by employing recently developed methods of inducing endomixis (Sonneborn, 1937) and of obtaining lines with long interendomictic intervals (Sonneborn, 1938). To induce endomixis, the surplus animals from daily isolation lines of cultivation were collected in a small amount of fresh culture medium and kept at 31° C. for a few days until endomixis occurred. To obtain lines with long interendomictic intervals, daily isolation lines which went into endomixis were replaced by sister lines which had not yet gone into endomixis. In this way, lines with long intervals are selected for study while the lines with shorter intervals are eliminated.

Using these techniques, the following experiment was performed. The vegetative descendants of a single endomictic individual were cultivated as 24 daily isolation lines of descent for 165 days. During this time, all lines that went into endomixis were eliminated and re-

[1] This work was suggested by Dr. T. M. Sonneborn, to whom I wish to express my sincere appreciation for his helpful advice and assistance.

placed by surplus animals from sister lines; so that at all times this group (I, Fig. 1) consisted of 24 lines that had not been in endomixis since the start of the experiment. At five successive intervals of 21 to 31 days, surplus animals from this group were induced to go into

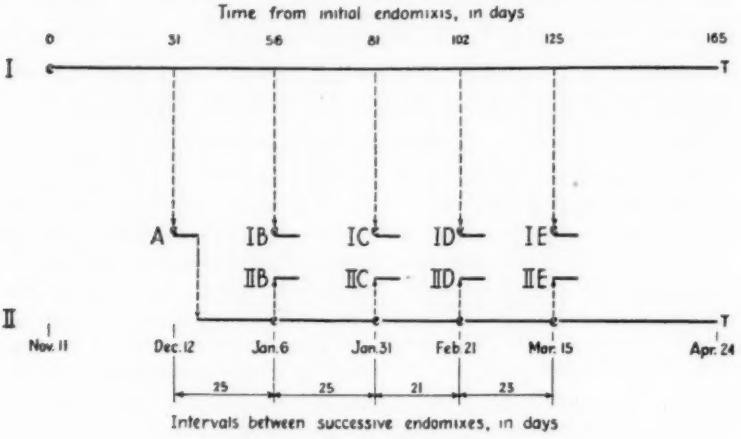

FIG. 1. Plan of Experiment

The horizontal lines (solid) represent groups of isolation culture lines. Long, solid line "I" represents a group of 24 isolation lines from which all endomictics were eliminated. Long, solid line "II" represents a group of 24 isolation lines, interrupted periodically (at points marked *e*) for the induction of endomixis in small mass cultures. The short, solid, horizontal lines represent groups of isolation lines carried for 15 generations. The length of the short lines corresponds to the average number of days that the 15 generations lived in all the groups.

The vertical lines (broken) connect the source groups (represented by long, solid, horizontal lines) with their derived groups (represented by short, solid, horizontal lines). In each case, the derived group began with animals in endomixis taken from mass cultures of animals from the source group.

A, *B*, *C*, *D*, and *E* are the designations given to the derived groups from both I and II. The four derived pairs of groups of isolation lines between which comparisons are made are groups I_B, II_B; I_C, II_C; I_D, II_D; I_E, II_E. Group II was derived from Group I_A by continuing, without endomixis, 24 of the lines of this group.

e stands for climax of endomixis. This symbol is put in on the day on which endomictics were isolated from the mass cultures.

T indicates the time of termination of the experiment.

Time in days, from the initial endomixis in Group I, is shown on the horizontal axis at the top of the diagram. The number of days between successive endomixes is shown at the bottom of the diagram.

endomixis and the resulting mortality in them was determined. In these groups (I_A, I_B, I_C, I_D, and I_E, Fig. 1), therefore, the mortality after interendomictic intervals of 31, 56, 81, 102, and 125 days, respectively, was ascertained. At the same time that each of these

groups (except the first) was being studied, there was examined a control group of lines that went through endomixis at the same time, but had instead an interendomictic interval of normal extent (21 to 25 days). The four control groups (II_B, II_C, II_D, and II_E) were obtained by inducing four successive endomixes at the proper time in descendants of the group I_A. Comparisons of mortality were thus made between the following pairs of groups: I_B (previous interval 56 days) with II_B (previous interval 25 days); I_C (previous interval 81 days) with II_C (interval 25 days); I_D (interval 102 days) with II_D (interval 21 days); and I_E (interval 125 days) with II_E (interval 23 days).

In this experiment, the animals employed were all descended vegetatively from one which was isolated from a Johns Hopkins stock

TABLE I

Number of fissions from climax of endomixis until death in sixty non-viable lines among one hundred and twenty exendomictic lines followed through until death or the next induction of endomixis.

(In these lines, the maximum interendomictic interval was 71 fissions.)

Number of Fissions until Death	Frequency
1	2
2	3
3	4
4	6
5	13
6	7
7	7
8	6
9	6
10	1
14	1
27	1
29	1
30	1
37	1

mass culture of Woodruff's long-lived Yale race. The organisms were cultivated throughout according to the methods described by Sonneborn (1936). Samples of all lines, except those not carried through till the next endomixis, were stained daily to determine the nuclear condition.

Relationship of the Interendomictic Interval to the Percentage of Mortality after Endomixis

The percentage mortality was computed as follows: In each group, each endomictic animal was cultivated as a single daily isolation line until the fifteenth fission after the climax of endomixis. The per-

centage of lines that died during this period was the percentage mortality for the group. The period of 15 fissions was decided upon as a conservative standard in view of the facts that the process of endomixis itself lasts not more than 9 or 10 fissions and that experience of previous investigators (e.g. Caldwell, 1933) as well as our own showed that deaths rarely occur in the interval from the tenth generation after the climax of one endomixis until the following endomixis. Table I gives the frequency of death at various stages of the interendomictic interval in the material here investigated. As appears from the table, deaths occurred but rarely after the tenth fission.

The results of the experiment are summarized in Tables II to IV. Table II gives a general view of the relation of mortality after endomixis to the length of the previous interendomictic interval. As appears in the table, the percentage mortality increases as the previous interendomictic interval increases. It rose from 32.2 per cent after

TABLE II

Relation of mortality after endomixis to length of previous interendomictic interval.

Previous Interendomictic Interval in Days	Percentage of Mortality after Endomixis	Number of Endomictic Lines Observed
21	32.2	90
23	62.5	48
25	59.3	91
25	57.0	79
31	66.6	48
56	71.4	49
81	79.2	53
102	89.3	84
125	100.0	29

an interval of 21 days to 100 per cent after an interval of 125 days. The first five intervals, 21, 23, 25, 25, and 31 days are normal interendomictic intervals for Woodruff's stock of *Paramecium aurelia*. The last four intervals of 56, 81, 102, and 125 days are abnormally long intervals for this stock. It will be noted that even in normal interendomictic intervals the percentage of mortality was high.

It will be observed, from an examination of Table II, that there may be great differences in mortality after endomixis even when the intervals between endomixes are practically the same. Such a difference appears between the percentages of mortality after the 21- and 23-day intervals when the percentages of mortality were 32.2 per cent and 62.5 per cent, respectively. Obviously there are factors other than interendomictic interval involved in the production of mortality. Environmental factors probably play an important rôle here. Therefore, it

was essential, for the purposes of this study, to make simultaneous comparisons of groups kept under identical cultural conditions.

The results of such simultaneous comparisons are given in Table III. There the percentages of mortality from endomixis after four abnormally long interendomictic intervals are compared with the percentages of mortality from concurrent endomixes after four normal intervals. Thus, when the interendomictic interval was 56 days, there was 71.4 per cent mortality, as compared with 59.3 per cent in the concurrent group with a normal interval of 25 days. When the interval was increased to 81 days, the mortality was between 79.2 and 84.0 per cent (the exact figure depending upon how many of 20 animals that died without dividing—and hence without a determination of whether they had been in endomixis—were in endomixis), as compared with

TABLE III

Relation between length of previous interendomictic interval and percentage of mortality after endomixis.

Groups with Normal Interendomictic Intervals			Groups with Long Interendomictic Intervals		
Name of Group	Previous Interendomictic Interval in Days	Percentage Mortality after Endomixis	Percentage Mortality after Endomixis	Previous Interendomictic Interval in Days	Name of Group
II_B	25	59.3	71.4	56	I_B
II_C	25	57.0	79.2	81	I_C
II_D	21	32.2	89.3	102	I_D
II_E	23	62.5	100.0	125	I_E

57.0 per cent in the concurrent group with a normal interval of 25 days. When the interval was still greater, 102 days, the mortality likewise increased to 89.3 per cent as compared with 32.2 per cent in the controls with a normal interval of 21 days. Finally, when the interval reached 125 days, the mortality was 100 per cent, as compared with 62.5 per cent in the controls with a normal interval of 23 days. The effects of still greater intervals could not be studied because efforts to induce endomixis at intervals of 148, 158, and 161 days all failed; all but a few of the animals in the induction cultures died within 48 hours.

The results of the experiment, as summarized in Tables II and III, show clearly that as the time between two successive endomixes increases, the percentage of lines that are unable to survive the second endomixis also increases until eventually no animals can survive.

Relationship of the Interendomictic Interval to the Number of Generations between Endomixis and Death

The relationship between the length of the previous interendomictic interval and the number of generations which the non-viable exendomictic lines lived after the climax of endomixis is shown by the data in Table IV. When the previous interval was of normal extent, i.e. about 25 days, as was the case in the control groups, the mean number of generations which the lines lived after the climax of endomixis remained fairly constant, ranging only from 5.0 to 5.9 generations.

TABLE IV

Length of life in number of generations from climax of endomixis until death, in the non-viable exendomictics, in relation to prior interendomictic interval. (The number dying without fission was not determined in experiments 1 and 2.)

Experiment	Group	Interendomictic Interval in Days	Number of Generations from Climax of Endomixis until Death																Total Non-viable Exendomictics	Mean
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1	II_B	25	?	1	2	3	5	15	7	8	8	4	1						54	5.9
	I_B	56	?	1	1	7	9	5	6	3	2			1					35	4.8
2	II_C	25	?	1	1	5	6	13	7	4	2	1	1	2		1	1		45	5.8
	I_C	81	?	7	3	3	6	9	8	4	1	1							42	4.4
3	II_D	21	3	1	2	3	3	3	7	2	1	1	2	1					29	5.0
	I_D	102	6	1	5	13	18	13	8	4	1	4	2						75	4.4
4	II_E	23	4		3	4	3	5	1	2	2	1	1		1		2	1	30	5.5
	I_E	125	21	1	2	2	2				1								29	0.9

Among the experimental groups the mean number of generations decreased from 4.8 generations when the interval was 56 days to 0.9 generation when the interval was 125 days.

In contrast to the steady increase in mortality rate with increasing interendomictic intervals, the mean survival period shows no such steady change. When the interendomictic interval was twice as great as normal the survival period decreased but little. Even when the interendomictic interval was 3 or 4 times as great as normal, the survival period was decreased, on the average, by only about one fission.

Differences of this magnitude are of doubtful significance because the method of determining when the climax of endomixis occurred involved an uncertainty. No direct observation could be made on the endomictic animals removed from the induction cultures to begin the experimental and control groups; but the next day one or more products of their fission were stained and the nuclear condition recorded. On the basis of the number of fragments of the old macronucleus, their size and the intensity of stain, and the size, form, and intensity of stain of the new macronucleus or its anlage, an estimate could be made of how many fissions had occurred since the climax of endomixis; but such an estimate may often be in error by one or two fissions. For this reason, little significance is attached to the slight differences among the preceding groups in the mean number of generations that the non-viable lines survived.

In the group with the longest interendomictic interval, however, the difference is so great as to be unquestionably significant. After an interendomictic interval of 125 days, the non-viable exendomictics went through only one-sixth as many fissions as the non-viable exendomictics with normal prior interendomictic intervals. Indeed, 72.4 per cent of them failed to divide at all after the climax of endomixis. On the third day without fission they were all stained and found to contain fragments of the old macronucleus, but no new anlage or macronucleus. It might be suggested that about three-fourths of the parent lines had lost their micronuclei before endomixis was induced. In such animals only the destructive phases of endomixis can take place as there is no reserve micronucleus from which a new macronucleus can be formed. Loss of the micronucleus after long omission of endomixis has in fact been observed by others in this laboratory (unpublished).

Discussion

1. In view of the complex and superficially paradoxical relations between endomixis and mortality, it has sometimes been held that endomixis is neither a definite phenomenon nor a normal one, but that it is a pathological response of the organism to adverse conditions, the type of response and its consequences varying with the degree of unfavorableness of the environment. In the present work, effects due to differences of environment were avoided by systematically exchanging culture medium between the various groups compared and by restricting comparisons to groups examined at the same time under the same cultural conditions. Thus, the differences in mortality after endomixis were not consequences of environmental differences. Nor were they due to the cumulative action of unfavorable conditions, for in

each case the groups compared had been subjected equally long to the same conditions. The differences in mortality after endomixis must therefore have been due to the difference in the prior interendomictic interval. The fact that mortality is increased after longer intervals shows that the frequent occurrence of endomixis is an advantage to the stock. In view of this, the interpretation of endomixis as pathological is untenable. Similar conclusions were reached by Kimball (1937) from a study of the precise ratios in which sex segregates after endomixis in this species.

2. The view that Protozoa are potentially immortal and that natural death does not exist among them became popular during the last quarter of the nineteenth century. It has long been known, however, that while a race as a whole may be potentially immortal, certain members of the race are doomed, from internal causes, to die. Thus, Jennings (1913) and others showed that conjugation often resulted in unavoidable death. Erdmann and Woodruff (1916), Jennings, Raffel, Lynch, and Sonneborn (1932), Raffel (1932), Caldwell (1933), Sonneborn and Lynch (1937), and others have shown that endomixis likewise often results in death. Woodruff (1917), Sonneborn (1935), and Jennings and Sonneborn (1936) have shown that long omission of endomixis ultimately results in death. To these intrinsic causes of death in *Paramecium* the present paper adds another: The mortality at endomixis is directly proportional to the preceding interval without endomixis.

Summary

1. Using an interendomictic interval of 20 to 30 days as a standard, it was shown that intervals approximately two, three, four, and five times this long resulted in progressive increases in mortality after endomixis until 100 per cent mortality occurred.

2. At intervals greater than this, the animals died before endomixis could be induced under conditions favorable for its induction.

3. The mean number of generations which non-viable lines survived the climax of endomixis was 5.0 to 5.9 generations when the previous interval was of normal extent. After an interval of 125 days, survival dropped greatly to a mean of 0.9 generation.

4. The results are shown to disagree with current interpretations of mortality at endomixis based on the concept of endomixis as a pathological process.

5. The results show that unusually long interendomictic intervals are, like other previously known conditions, a cause of "natural death" in Protozoa.

LITERATURE CITED

CALDWELL, L., 1933. The production of inherited diversities at endomixis in Paramecium aurelia. *Jour. Exper. Zoöl.*, **66**: 371.

ERDMANN, R., AND L. L. WOODRUFF, 1916. The periodic reorganization process in Paramæcium caudatum. *Jour. Exper. Zoöl.*, **20**: 59.

JENNINGS, H. S., 1913. The effect of conjugation in Paramecium. *Jour. Exper. Zoöl.*, **14**: 279.

JENNINGS, H. S., DANIEL RAFFEL, RUTH STOCKING LYNCH, AND T. M. SONNEBORN, 1932. The diverse biotypes produced by conjugation within a clone of Paramecium aurelia. *Jour. Exper. Zoöl.*, **62**: 363.

JENNINGS, H. S., AND T. M. SONNEBORN, 1936. Relation of endomixis to vitality in Paramecium aurelia. *Comptes rendus du XII^e Congrès International de Zoölogie* (Lisbonne 1935), pp. 416–420.

KIMBALL, R. F., 1937. The inheritance of sex at endomixis in Paramecium aurelia. *Proc. Nat. Acad. Sci.*, **23**: 469.

RAFFEL, DANIEL, 1932. The occurrence of gene mutations in Paramecium aurelia. *Jour. Exper. Zoöl.*, **63**: 371.

SONNEBORN, T. M., 1935. The relation of endomixis to vitality in Paramecium aurelia. *Anat. Rec.*, **64**: (Supplement No. 1) 103.

SONNEBORN, T. M., 1936. Factors determining conjugation in Paramecium aurelia, I. The cyclical factor: the recency of nuclear reorganization. *Genetics*, **21**: 503.

SONNEBORN, T. M., 1937. Induction of endomixis in Paramecium aurelia. *Biol. Bull.*, **72**: 196.

SONNEBORN, T. M., 1938. The delayed occurrence and total omission of endomixis in selected lines of Paramecium aurelia. *Biol. Bull.*, **74**: 76.

SONNEBORN, T. M., AND R. S. LYNCH, 1937. Factors determining conjugation in Paramecium aurelia. III. A genetic factor: the origin at endomixis of genetic diversities. *Genetics*, **22**: 284.

WOODRUFF, L. L., 1917. The influence of general environmental conditions on the periodicity of endomixis in Paramecium aurelia. *Biol. Bull.*, **33**: 437.

THE EFFECT OF SHORTER THAN NORMAL INTER-ENDOMICTIC INTERVALS ON MORTALITY AFTER ENDOMIXIS IN PARAMECIUM AURELIA

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In the preceding paper, Pierson (1938) has shown for *Paramecium aurelia* that mortality after endomixis is directly proportional to the length of the interendomictic interval, when the latter is longer than normal. The present paper examines the same question when the interendomictic intervals are shorter than normal.

The same race of *Paramecium aurelia* is examined in both studies. All individuals in the present study were descended without endomixis from one taken from a stock mass culture of this race on February 22, 1936. The methods of culture and of induction of endomixis were similar to those employed by Pierson.

The experiment was performed as indicated in Fig. 1. From a single endomictic individual, 24 daily isolation lines of cultivation were followed for 28 days (Group I, Fig. 1). During this time, samples of each line stained daily showed that no endomixis occurred. On the eighth and sixteenth days after the initial endomixis, the surplus animals from the isolation lines were collected in a mass culture and placed at 31° C. In each case, a high percentage of individuals went into endomixis after 48 hours in these conditions. From each of these, a group of endomictic individuals was isolated and cultivated (Groups II and III, Fig. 1). On the twenty-sixth day after the initial endomixis, three mass cultures were set up, each consisting of the surplus animals from one of the three groups under cultivation. These cultures were placed at 31° C. and all contained numerous endomictic individuals two days later. From each of the three cultures a group of endomictic individuals was isolated and cultivated. These three groups of animals were thus all in endomixis at the same time, but they differed in the interval since the last preceding endomixis: in one, the interval was 10 days; in the second, 18 days; and in the third, 28 days.

The three groups will be designated the 10, 18, and 28-day groups, in reference to their prior interendomictic intervals. The 10-day

[1] The author is grateful to Dr. T. M. Sonneborn who suggested the problem and offered his advice throughout the experiment, and to Bernice Pierson and Nathaniel Finkelstein for their kind assistance.

group was begun with 192 individuals from the culture in which endomixis had been induced; but only 51 of these were in endomixis, as determined by staining products of their fissions on the next day. From each of these 51 individuals, a single daily isolation line was cultivated until it died or until 15 successive fissions had taken place. Of the 51 lines, 48 lived through the 15 fissions and three died after 5 to 12 fissions, giving a mortality rate of 5.9 per cent.

The 18-day group was begun with 144 individuals from the induction culture; but only 94 of these were in endomixis, as subsequently determined. Of these 94 endomictic individuals, 74 lived through the following 15 fissions, and 20 died after 1 to 7 fissions, giving a mortality rate of 21.3 per cent.

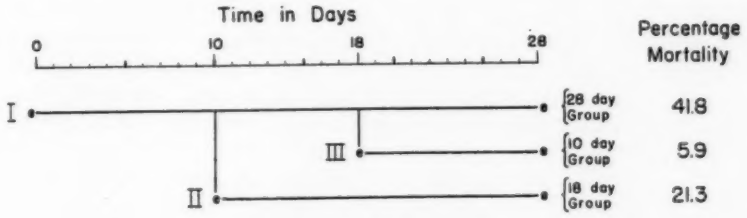

FIG. 1. Plan of Experiment

The three horizontal lines, I, II, and III, represent groups of isolation culture lines carried without endomixis for 28, 18, and 10 days, respectively. The vertical lines connect the source group (horizontal line I) with the two derived groups (horizontal lines II and III). In each case the derived group began with animals in endomixis taken from mass culture of animals from the source group.

e stands for endomixis.

The upper line shows the time in days since the initial endomixis in Group I.

The percentage mortality following endomixis in each group is shown to the right of the line representing that group.

The 28-day group was begun with 144 individuals from the induction culture. Of these, 110 were shown by subsequent staining of their descendants to have been in endomixis. These 110 endomictic individuals were cultivated in the same way as were those of the other two groups. In this group, 64 lived through the 15 fission period of observation and 46 died after 0 to 13 fissions, giving a mortality rate of 41.8 per cent.

Thus, the group with a normal interendomictic interval of 28 days suffered a mortality rate of 41.8 per cent after endomixis, as compared with 21.3 per cent and 5.9 per cent mortality following abnormally short interendomictic intervals of 18 and 10 days, respectively.

The results therefore extend those of Pierson to include abnormally

short as well as abnormally long interendomictic intervals. Throughout the entire range of intervals investigated, the mortality after endomixis is directly proportional to the extent of the preceding interendomictic interval.

LITERATURE CITED

PIERSON, BERNICE F., 1938. The relation of mortality after endomixis to the prior interendomictic interval in Paramecium aurelia. *Biol. Bull.*, **74**: 235.

DIPLOIDS FROM UNFERTILIZED EGGS IN HABROBRACON

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FEMALES

The occurrence of impaternate females (females from unfertilized eggs) in the parasitic wasp *Habrobracon juglandis* (Ashmead) has previously been reported (Speicher, 1934). Such females occur sporadically from various virgins, and regularly constitute about 1 per cent of the F_2 population from F_1 virgins produced by outcrossing females from tapering or reverted tapering stocks. The hypothesis was offered that these diploid impaternate females might be produced by the failure of the second maturation division in the unfertilized egg and, carrying two chromatids from a single tetrad, would provide material for studying the mechanics of crossing-over.

When F_1 virgins were heterozygous for recessive factors, F_2 impaternate females occurred in the ratio of one homozygous dominant, to two heterozygotes, to one homozygous recessive for each locus involved. This 1 : 2 : 1 ratio was at variance with results obtained in other organisms where more than one strand may be recovered from a single tetrad, notably *Drosophila* (Anderson, 1925) and *Neurospora* (Lindegren, 1933). Here the first maturation division is reductional at the spindle fiber and in dyads from that division the homozygosis of any locus depends upon the amount of crossing-over between it and the fiber, and hence is a function of its location along the chromosome. In *Habrobracon*, except for the locus of the sex-linked factor fused (Whiting and Speicher, 1935), the amount of homozygosis was consistently 50 per cent for all loci tested, even including those of two recessives known to be linked and separated by a distance of ten units.

It was realized from the beginning of the work that other hypotheses could be advanced to explain the formation of impaternate females. And it was apparent that the 1 : 2 : 1 ratio obtained would be expected for all loci if the two homologous chromosome strands of an impaternate female came from two tetrads, independent and com-

[1] The authors are indebted to Professor P. W. Whiting of the University of Pennsylvania for the use of special microscopic equipment which was supplied in part by a grant to him from the Elizabeth Thompson Science Fund.

pletely reduced, rather than from a single tetrad which underwent only partial reduction. As an investigation of oögenesis in *Habrobracon* had already been begun (Speicher, 1936), it seemed best to suspend further genetic studies until the method of formation of impaternate females could be determined cytologically. Those findings are here reported for the first time.

Since impaternate females had been shown to be genetically diploid, whereas their sibs are haploid, a cytological analysis of their formation seemed practical. According to past work on other forms at least three basic hypotheses could be considered. First, one of the two maturation divisions of an unfertilized egg could be suppressed, as was observed by Silvestri (1908) in the wasp *Prospalta*. This would leave a cleavage nucleus containing the diploid number of chromatids, which would restore the diploid number of chromosomes if the homologues separated. Second, fusion of two reduced egg nuclei present

TABLE I

Progeny from No. 25/reta virgin females.

F_1 virgin ♀ ♀	1	2*	3	4	5	6	7*	8	9	10	11
Sons	83	0	91	26	95	97	5	67	29	113	63
Daughters	1	2	0	0	5	0	0	0	0	0	7
Eggs collected	49	3	50	36	46	61	11	29	48	56	51

* Died before completion of experiment.

in a binucleate egg also would restore the diploid condition. Third, the egg might originate as a tetraploid, undergo reduction and thus become diploid. A fourth hypothesis, the doubling of chromosomes in the haploid egg during cleavage, is eliminated because tests have shown that impaternate females may be genetically heterozygous.

The first two theories were tested together. Over 300 eggs were collected from virgin females produced by crossing reverted tapering and stock 25. They were fixed at first cleavage prophase and stained by the Feulgen whole-mount method. The same females produced collectively 15 impaternate daughters among 724 sons, over 2 per cent of the total. Among the eggs studied approximately the same proportion would be expected to show cytological differences if either theory were correct. Suppression of a maturation division would result in a decrease in the normal number of polar nuclei formed at the egg margin. A binucleate egg would be expected to show two groups

of polar nuclei, totaling twice the normal number. An examination of all eggs revealed none showing either of these two differences.

Treatment of the third hypothesis requires chromosome counts during maturation; accordingly it was necessary to obtain a new lot of eggs fixed at an earlier stage than the above. It had been noticed previously that some virgin females produced impaternate daughters in small groups while others in the same experiment produced no daughters whatever. Inclusion in the data of the offspring from the latter virgins lowers the percentage of impaternate females among total offspring. It was therefore possible to raise the expected percentage of exceptional eggs by selecting eggs only from virgins known to be producing impaternate daughters. This was accomplished as follows. Eleven females from a cross of reverted tapering by stock 25 were placed with host caterpillars. Eggs laid upon the caterpillars during seven consecutive days were fixed at first anaphase and temporarily stored in alcohol. Eggs laid at night over the same period were allowed to develop, in order to indicate which of the eleven females were thelytokous. Results are shown in Table I.

Fig. 1.

Fig. 2.

FIG. 1. First anaphase of normal egg. × 3,000.
FIG. 2. First anaphase of tetraploid egg. × 3,000.

Seven females, producing a total of 428 sons, had no daughters, while the remaining four produced 241 sons and 15 daughters. The 149 eggs collected from the latter four females were then stained by the Feulgen whole-mount technique. One hundred and ten of these were in condition to study; the remainder were either lost in handling or were collapsed. Ninety-eight eggs were unquestionably diploid, seven were unquestionably tetraploid and five more were questionably tetraploid. The clear cases of tetraploidy showed twenty chromosomes, presumably bivalents although individual chromatids have never been observed in *Habrobracon* oögenesis due probably to their small size, moving to one or to each pole. Normal diploid eggs show only ten chromosomes going to each pole, Figs. 1 and 2.

These cytological data, and the fact that impaternate females come in groups from certain mothers suggest the probability that production of tetraploid eggs, as developed from patches of tetraploid

ovarian tissue, is responsible for the appearance of diploid impaternate females among haploid brothers.

Males

Diploid males of biparental origin have been reported repeatedly in *Habrobracon*. Since diploid females are produced by virgins as a result of tetraploidy in egg cells, it seems plausible that diploid impaternate males may be produced in the same way. According to Whiting's scheme of sex-determination in *Habrobracon* (Whiting, 1933) those eggs which were diploid after reduction and contained sex chromosomes *X* and *Y* would produce impaternate females, while those which contained chromosomes *XX* or *YY* would produce diploid impaternate males. If distribution of chromatids is random the number of diploid females and males produced should be equal.

Table II

Data from tests for diploid impaternate males.

F_1 ♀ ♀	F_2 haploid ♂ ♂, *A* or *a*	F_2 impaternate ♀ ♀		
		AA	*Aa*	*aa*
o^1/o^4	6222	6	10	5
Le/le	6070	17		6
St/st	4408	4	2	0

Three experiments were set up in an effort to produce diploid impaternate males that could be distinguished genetically from their haploid brothers. Since the highest percentages of impaternate F_2 diploids had previously resulted from outcrosses of tapering and reverted tapering females, tapering was again used as the maternal stock in one experiment. In the other two it was necessary to introduce recessive factors into the cross through the females. In order to insure the occurrence of thelytoky in these cases the recessives were repeatedly bred up to reverted tapering, and a stock related to it but having the desired genetic constitution was then derived and supplied the females for the parental crosses. In each experiment F_1 virgins were produced carrying both of two alleles that give rise to a distinct phenotype when they are heterozygous.

Results are given in Table II where the first column shows the pairs of allels used to make the heterozygotes recognizable phenotypically, the second column gives the number of normal haploid males in the F_2, and the third column gives the classes of impaternate females. Theo-

retically diploid impaternate males should have occurred in the same numbers and in the same genetic ratio (1 homozygous dominant, *AA* : 2 heterozygotes, *Aa* : 1 homozygous recessive, *aa*) as did the impaternate females, although among the males only the heterozygotes (*Aa*) would have been distinguishable from their normal haploid brothers.

No diploid males were found and the experiments are published at this time because the stocks with which they might be repeated are no longer extant and the work is finished unless or until the thelytokous tendency reappears in *Habrobracon*. However, results are not considered entirely convincing, nor is the evidence against the occurrence of diploid impaternate males considered conclusive. The following paragraphs tell for each experiment why the tests were not as satisfactory as had been expected.

o^{i}/*o*^{d}.—Wasps heterozygous for the allelomorphic eye colors ivory (*o*^{i}) and dahlia (*o*^{d}) had previously been distinguished phenotypically by a lighter color than the dark red of homozygous dahlia (Torvik, 1931). In this experiment light dahlia was recognizable in the F_2 females, where it was checked by breeding tests. In the F_2 males, however, the color varied widely, grading from dark to light. Forty males were recorded as having eyes as light as their ten heterozygous sisters.

F_1 virgins in the eye color experiment were also heterozygous for the sex-linked factor fused (*fu*, antennæ and tarsi). Diploid impaternate daughters would be heterozygous for the sex region (*XY*), and also for fused except where cross-overs had occurred betewen fused and the sex region (10 per cent of the cases). Of the 21 impaternate females produced none was homozygous fused, and wherever it was possible to make breeding tests fused males occurred among their progeny, so that actually no cross-over cases were found. Diploid impaternate males, on the other hand, would be either *XX* or *YY*, and homozygous for *fu* or *Fu* except where cross-overs had occurred. Thus almost half the diploid males would carry two fused genes. C. H. Bostian had previously noted that the presence of two fused genes in diploid biparental males frequently made the antennæ shorter than those of fused haploid males where only one fused gene occurs. It was hoped that here extreme shortness of antennæ might serve as an additional tag for diploidy. However, antennal length proved to be too variable to be of any service, grading gradually from long to short.

Le/*le*.—In heterozygotes for the gene lemon (*le*, body color) pigmentation is normal except for the basal segments of the antennæ which are yellow instead of dark brown. The dominant factor Min-

nesota yellow (*My*, base of antennæ) produces exactly the same phenotype at high temperatures. It is homozygous in reverted tapering stock, and an effort was made to breed it out before this experiment was begun, but its presence was simply hidden by temperature changes. In the F_2 both *My* and *le* segregated. Of the impaternate females with normal body color it was impossible to tell which carried lemon. All but one were recorded as having yellow antennal bases due to the presence of one or the other or both of the mutant genes. If diploid males heterozygous for lemon were present, it was impossible to distinguish them from their haploid Minnesota yellow brothers.

St/st.—The experiment using the stumpy gene (*st*, legs) was abandoned because the number of F_2 impaternate females was so small that the expectancy for heterozygous diploid males fell so low as to make the search unprofitable, in spite of the fact that this was the one case where parental females had been of pure tapering stock.

Summary

1. Cytological evidence shows that unfertilized eggs which give rise to females are tetraploid before reduction and therefore diploid after reduction.

2. No diploid males from unfertilized eggs were found among 16,700 males examined.

LITERATURE CITED

Anderson, E. G., 1925. Crossing over in a case of attached X chromosomes in Drosophila melanogaster. *Genetics*, **10**: 403.

Lindegren, Carl C., 1933. The genetics of Neurospora—III. Pure bred stocks and crossing-over in N. crassa. *Bull. Torrey Botanical Club*, **60**: 133.

Silvestri, F., 1908. Apunti sulla Prospalta Berlesei, How. e specialmente sui primi stati del suo sviluppo. *Boll. Lab. Zool. R. Sc. Agr. Portici*, **3**: 22.

Speicher, B. R., 1936. Oögenesis, fertilization and early cleavage in Habrobracon. *Jour. Morph.*, **59**: 401.

Speicher, Kathryn Gilmore, 1934. Impaternate females in Habrobracon. *Biol. Bull.*, **67**: 277.

Torvik, Magnhild M., 1931. Genetic evidence for diploidism of biparental males in Habrobracon. *Biol. Bull.*, **61**: 139.

Whiting, P. W., 1933. Selective fertilization and sex-determination in Hymenoptera. *Science*, **78**: 537.

Whiting, P. W. and Kathryn Gilmore Speicher, 1935. Impaternate daughters of females heterozygous for a sex-linked gene in Habrobracon. *Am. Nat.*, **69**: 82.

INACTIVATION OF SPERM BY X-RADIATION IN HABROBRACON

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Sterility of the male may be due to lack of sperm, to defective or inactivated sperm unable to fertilize the eggs or to sperm carrying dominant lethals which kill the fertilized eggs.

The wasp *Habrobracon* is convenient material for the study of these types of male sterility. Normally males develop from unfertilized eggs by haploid parthenogenesis, females from fertilized eggs. Absence or inactivation of sperm would result in the production of no daughters and of sons equal in number to those of unmated females. If all the sperm contain dominant lethals no daughters are produced and the fecundity of the mated females is correspondingly reduced. Consequently analysis of differences in biparental ratio and in fecundity indicates the type of male sterility which has been operative.

P. W. Whiting (1937) demonstrated that treatment of sperm with X-ray dosages of 20,000, 40,000 and 75,000 r units produces at least one dominant lethal in every sperm cell. No daughters occur in the progeny and the average number of males produced per day does not equal that to be expected from virgin females, indicating that many eggs are fertilized and die. Sperm treated with 75,000 r units fertilized almost as many eggs as untreated sperm; therefore the treatment apparently did not cause inactivation. However, a slight increase in the average males per day from mates of males treated with 75,000 r units as compared with those from mates of untreated controls suggested the possibility that spermatogenesis might to some extent be stopped and sperm supply decreased.

An experiment carried out with the X-ray equipment at the Marine Biological Laboratory, Woods Hole, Mass., July–August, 1937, was designed to test this hypothesis (Table I). One group of wild-type males (stock 32) was treated with 41,000 r units, another group with 142,000 to 143,000 r units. A third group was untreated. Each male was mated on each successive day to a different female which was

[1] The author is indebted to the University of Pennsylvania for furnishing laboratory space at the Marine Biological Laboratory during the summer of 1937, and to the Committee on Effects of Radiation on Living Organisms (National Research Council) for technical assistance furnished from a grant to Dr. P. W. Whiting.

segregated for breeding. Unmated females of the same stock were segregated each day as controls. The total number of days of the egg-laying periods of all females of any one group mated on the same day is the basis of the averages of offspring per day.

It was expected that successive matings would exhaust the sperm of the treated males provided that no spermatogenesis took place after the treatment. Such exhaustion does not occur since the last matings of these males produce average males per day equivalent to those from mates of untreated males and lower than the average for the offspring of unmated females.

TABLE I

Offspring from orange-eyed females (stock 11–o) mated with untreated or with x-rayed wild-type males (stock 32) or bred unmated.

Day of mating	Offspring from untreated males					Offspring from 41,000 r males			Offspring from 142,000 and 143,000 r males		
	Days	+ ♀ ♀	Per day	o ♂ ♂	Per day	Days	o ♂ ♂	Per day	Days	o ♂ ♂	Per day
1st	152	524	3.44	247	1.62	139	184	1.32	219	464	2.11
2nd	136	671	4.93	203	1.49	129	257	1.99	208	368	1.76
3rd	145	337	2.32	116	.80	120	245	2.04	120	285	2.37
4th	168	487	2.89	174	1.03	137	223	1.62	89	176	1.97
5th	113	303	2.68	103	.91	120	128	1.06	41	71	1.73
6th	93	185	1.99	62	.67	128	112	.88			
7th	117	302	2.58	59	.50	60	44	.73			
8th	100	258	2.58	87	.87	128	194	1.51			
9th and 10th	59	188	3.18	82	1.38	116	128	1.10			
Totals from mated females	1083	3255	3.01	1133	1.05	1077	1515	1.41	677	1364	2.01

Total from unmated females.... Days, 344. Males, 1073. Males per day, 3.12.

In the total averages of males per day for all the fraternities from treated, evidence is found of failure of sperm to fertilize and thus kill some of the eggs. The total average of males per day increases with increasing dosage and for 142,000 to 143,000 r units is intermediate (2.01) between the mated controls (1.05) and the unmated controls (3.12).

Inactivation of sperm following higher dosages rather than partial exhaustion of sperm supply due to partial inhibition of spermatogenesis is indicated by the fact that there is no increase in males per day from the later matings. It is probable that even much weaker dosages than those here used completely stop spermatogenesis. Thus

we have partial male sterility due to dominant lethals at relatively weak dosages, complete male sterility due to dominant lethals at stronger dosages, and partial sperm inactivation at very high dosages.

The average offspring per day (males) from unmated females (3.12) is less than the average offspring per day (females and males) from females mated to untreated males (4.05). This is attributed to recessive lethals segregating in certain fraternities summarized together in Table I. In other experiments viability of males and females has been about equal.

LITERATURE CITED

WHITING, P. W., 1937. Habrobracon as a means of testing the effectiveness of physical agents in causing mutations. *Proc. Penna. Acad. Sci.*, **11**: 50.

THE SEX RATIO IN MELITTOBIA CHALYBII ASHMEAD, GAMETOGENESIS AND CLEAVAGE IN FEMALES AND IN HAPLOID MALES (HYMENOPTERA: CHALCIDOIDEA)

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Introduction

In the chalcid-fly, *Melittobia*, females develop only from fertilized eggs, males from unfertilized eggs. The total number of males produced by any individual female is approximately only 3 per cent of the total number of offspring—an unusual condition in view of the fact that these males are indispensable for the continued existence of the species. Not only is the proportion of males very low, but even in virgin females, the number of eggs which develop parthenogenetically into males is not, it is estimated, much over 3 per cent of the total number of eggs laid. The remainder of the eggs die and shrivel up.

When an examination revealed that the males in this species are haploid and that we were not dealing with the common type of facultative parthenogenesis which involves a restoration of diploidy in a limited number of azygous embryos, the question that naturally presented itself was: Why does only such a small percentage of eggs possess parthenogenetic potentialities? As a step in the investigation of this problem cytological observations on gametogenesis, fertilization and cleavage are here recorded.

Life History

Melittobias have been much studied both in America and in Europe. Interest in them has centered about their life-history, their polyphagous habit and the striking structural antigeny they exhibit. More recently (Schmieder, 1933), polymorphism and the factors responsible for this phenomenon have been described in females of *M. chalybii*.

These parasites are most frequently found within the cocoons of certain wasps, especially *Sceliphron*, *Chalybion* and *Trypoxylon*. In addition to *M. chalybii*, taken in southern New Jersey, two other species, as yet unidentified, have been studied, one from New Jersey, the other from numerous localities in New Jersey, Pennsylvania, Ohio and Indiana. None of these three species when crossed with one an-

other produced any female offspring but only matroclinous males. The observations in this paper are all based on *M. chalybii* material. The other species were, however, also studied and were found to be, cytologically, like *chalybii*.

In the rearing of these insects, one mated female is placed on a host which has been removed from its cocoon. Feeding and oviposition soon commence and the first 12 to 30 female offspring that emerge oviposit on the same host. Finally, the remainder of the first and the entire second generation (altogether some 500 to 800 offspring) emerge. From 2–5 per cent, usually about 3 per cent, of these are males. Although the proportion of males is small and although their number is still further reduced by the mortal combats which occur among them, practically all the females are eventually fertilized. Indeed, the sex ratio here seems to be an adaptive feature which conserves the food supply of the species for the almost exclusive use of the female sex, the sex which alone serves the dispersal of the species. This method of rearing probably approximates what occurs in nature, except that in nature occasional cross-breeding between different lines no doubt may occur while in the laboratory inbreeding is the rule. The stock now on hand is the seventh successive inbred culture. The number of the generation is not known because of the intermingling of two successive generations in each culture. Of one of the other species, with a shorter life history, the twenty-sixth inbred generation is at hand.

Unmated females, segregated during the pupal stage, either may not oviposit at all or may lay only a very few eggs, perhaps from 1 to 5. Of these eggs one or two may develop into males, or all the eggs may die. In an exceptional instance 30 males were obtained from 2 virgins, but the record states that many dried up eggs were also found in this culture.

While virgin females are disinclined to oviposit, such females, after being mated with a male of a different species, generally oviposit as freely as do normally mated individuals. Apparently the act of mating or the presence of sperm in the seminal receptacle acts as a stimulus to oviposition. In no instance, however, did any females develop from such eggs, but only matroclinous males. In mating males of another species to *chalybii* it is necessary to employ the short-winged polymorphic form of *chalybii* whose copulatory reaction time corresponds more closely to that of the foreign males. This short-winged form, however, deposits eggs in very rapid succession and, even when normally mated, an unusually large proportion of these eggs fails to develop.

In the other two species studied no such difficulty exists and in them

it is evident, as in *chalybii*, that only a small proportion, possibly again not more than 3 per cent, develops parthenogenetically into males while all the other eggs die. In one instance 3 females produced over 200 eggs by the sixth day. They were allowed to continue to oviposit until the tenth day but this culture eventually yielded a total of only 20 males. These same females transferred successively to two other hosts, upon which oviposition continued at almost the initial rate, produced on the second host 16 males and on the third host 16 males. The same procedure when carried out with two other females yielded 12 males from the first, 6 from the second and 3 from the third host. No female offspring appeared in any of these cultures.

It is therefore evident that both virgins and females mated to males of another species may produce about the same number of male offspring as do normally mated individuals. Those of their eggs which fail to develop, one is tempted to assume, are those which would have yielded female offspring, had their mothers been mated in the normal way. Without a more adequate statistical analysis, it is not possible to make more definite statements at the present time, especially in view of the high egg mortality often observed even in normal cultures.

Materials and Methods

The *chalybii* material examined consists of: 50 pairs of testes of larvæ and pupæ, which were dissected out on glass slips and fixed as smear preparations with Carnoy-Lebrun's and Bouin's fluids; 25 pairs of testes, dissected out in Belar's solution, fixed mainly in Benda's, Bouin's and Petrunkevitch's fluids and sectioned at 4 to 6 μ in paraffin; 28 pairs of ovaries of pupæ and imagines, dissected out and fixed directly on glass slips with Carnoy-Lebrun's or Bouin's solution; 220 eggs of normally mated females and 154 eggs of females mated with males of another species, all fixed in Carnoy-Lebrun's medium.

The sectioned material was stained with Heidenhain's hæmatoxylin. The smears of testes and of ovaries were stained with Mayer's hæmalum, containing 2 per cent of acetic acid, and by the Feulgen method. For the staining of entire eggs the Feulgen method only was entirely satisfactory. All material was mounted in gum damar.

Observations on Males

Somatic Tissues

In the cells of the nervous system and of the imaginal discs of pupæ and prepupæ of males there were invariably 5 chromosomes, the haploid number (Figs. 1 and 2). This same number was again found in the unfertilized eggs in all stages from early cleavage until hatching (Fig. 13).

Testis

Development of spermatogonial cysts and multiplication of spermatogonia occur during larval life and only during that period are gonial division stages in evidence. In very young pupæ, in which not even the eye spots as yet show any traces of pigmentation, about one-third of the testis is already occupied by spermatids and mature sperm. Cysts containing the youngest spermatocytes (Fig. 3), arranged in the form of rosettes, occupy the anterior end of the testis.

The middle region is taken up largely by spermatocytes in the growth stage and by division figures. During the growth period the nucleus contains a prominent karyosphere and a lightly staining network (Fig. 4). This karyosphere gives way to the fine spireme of the first spermatocyte prophase. As the spireme finally becomes a very open one, the five chromosomes present can easily be distinguished in every cell (Fig. 5). In metaphase plates of spermatocyte divisions (Figs. 6 and 7), it is noted that all five chromosomes are atelomitic. The first division, most clearly recognizable in anaphase (Fig. 8), is abortive and yields only one second spermatocyte. In Fig. 8 and in the figures of the second spermatocyte division (Figs. 9 and 10), only the spindles are shown, which are always clearly distinct from the poorly fixing zone of cytoplasm which surrounds them. The two spermatids formed by division of each second spermatocyte both transform into sperm cells.

Observations on Females

Somatic Tissues

The cells of the nervous system and of the imaginal discs of female prepupæ are always diploid, with 10 chromosomes. The same is true of the follicular cells of the ovary and of the cells of embryos developing from fertilized eggs (Figs. 21 and 34).

Ovary

At the cephalic end of each of the 6 to 8 ovarioles comprising each ovary there is a region in which the oögonia and the future follicle and nurse cells are indistinguishable (Fig. 16). Immediately caudad of this limited region the young oöcytes become recognizable by the synaptic stages which are observable in them at a time when these cells are hardly larger than the neighboring presumptive nurse and follicle cells (Fig. 16). In the post-synaptic spireme (Fig. 22), 5 pachytene threads are always clearly discernible. Following synapsis, the oöcyte undergoes a steady, continuous growth until maturity,

while the follicle cells and the nurse cells assume the arrangement characteristic of meroistic ovarioles (Figs. 17–20). During this period of growth the chromosomes become more and more deconcentrated and diffuse (Figs. 23–25). In the course of this change they become at first less sharply outlined and open up, the opening up process beginning near their ends, in a manner suggesting a longitudinal splitting. Finally large open figures are formed which are most frequently in the form of loose rings. Although this ring-like organization is not always determinable for each of the chromatic elements due to their large size, haziness and frequent discontinuity, yet it is evident that the chromosomes never entirely disappear during the growth period of the egg.

Toward the end of the growth period the diffuse chromosomes of the germinal vesicle condense and contract into small chromatic bodies which are perhaps suggestive of the precociously formed abortive maturation spindle often described in the literature. (This stage will require additional study.) Finally, when the egg is ready to leave the oviduct, the nucleus is represented by a single small karyosphere

Explanation of Figures

Figures 3, 4, 8, 9 and 10 are from material fixed with Benda's fluid and stained with Heidenhain's hæmatoxylin.

Figure 6 is from material fixed with Bouin's fluid (B 15) and stained with Heidenhain's hæmatoxylin.

All other figures, on both plates, are from material fixed with Carnoy-Lebrun. Figures 16, 17, 21–24 are from slides stained with acid hæmalum; the remainder from slides stained by the Feulgen method.

Magnification: All figures of individual cells or nuclei (Figs. 1–11, 13, 14, 21–26, 28, 30, 32, 34), × 5,000.

Figures of eggs (Figs. 12, 15, 27, 29, 31, 33, 35), × 500.

Figures of the ovariole (Figs. 16–20), × 400.

Plate I

Fig. 1. Somatic cell of larva, male; late prophase.

Fig. 2. Somatic cell of larva, male; metaphase.

Fig. 3. Young spermatocyte, from a rosette.

Fig. 4. Spermatocyte in the growth stage.

Fig. 5. Spermatocyte in late prophase.

Figs. 6 and 7. Spermatocytes in metaphase.

Fig. 8. First spermatocyte anaphase.

Fig. 9. Second spermatocyte metaphase, lateral view.

Fig. 10. Second spermatocyte anaphase.

Fig. 11. Second oöcyte division, metaphase; from Fig. 12.

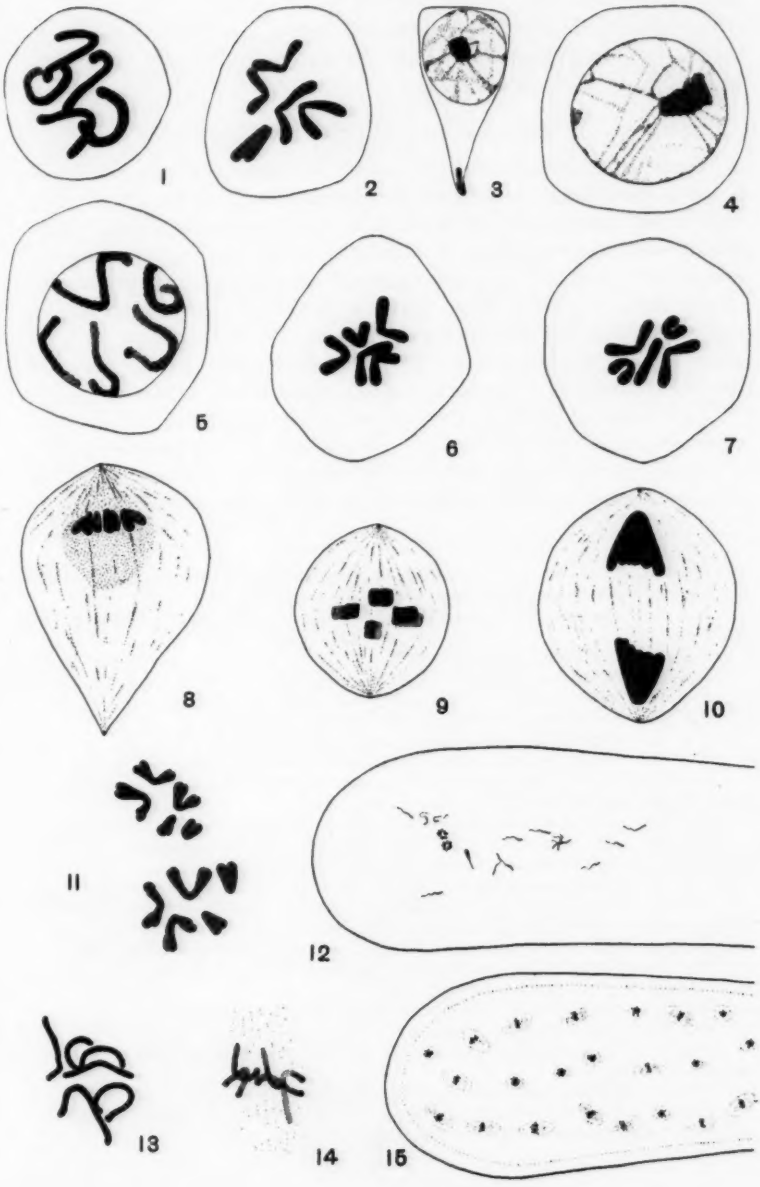
Fig. 12. Egg in second meiotic division, from a female mated with a male of another species. One sperm is within the egg, at the center, and is swollen; 14 additional sperm are adhering to the outer surface of the egg.

Fig. 13. Haploid metaphase of a cleavage nucleus, from Fig. 15.

Fig. 14. Haploid metaphase of a cleavage nucleus, lateral view, from Fig. 15.

Fig. 15. Anterior portion of an egg in the eighth cleavage division; haploid. Only those cells which occupy that half of the egg nearest the observer are represented in the figure.

PLATE I



separated from the deutoplasm of the egg by a thin zone of clear protoplasm (Fig. 20).

In Figs. 16 to 20 representative portions of a complete ovariole are shown, including those from which the nuclei, drawn on a larger scale in Figs. 21 to 25, were selected.

Cleavage in Unfertilized Eggs

Unmated females deposit only a very few eggs and of these few only a small percentage undergoes development. It is therefore more practicable to study haploid development in eggs obtained from females mated with another species of *Melittobia*, since after such mating oviposition occurs often at a rate comparable to that occurring in normally mated females. The foreign sperm is generally observed as having entered the egg but actual syngamy seems never to occur, or, at least, never to yield viable zygotes. The small percentage of eggs that eventually hatches always produces haploid males of the maternal type.

Eggs fixed ten minutes after oviposition are commonly in the second oöcyte stage, as shown in Fig. 12. Here is seen the foreign sperm which is within the egg and is considerably swollen, as well as an unusual number of additional sperm adhering to the outside surface of the egg.

Plate II

Fig. 16. Cephalic end of an ovariole, with undifferentiated cells, oöcytes in synizesis, and post-synaptic spireme; nurse cells and follicle cells.

Fig. 17. Portion of an ovariole with young, growing oöcyte and nutritive chamber.

Fig. 18. The same, somewhat older.

Fig. 19. Anterior end of a half grown egg, with the follicle cells.

Fig. 20. Anterior end of a fully grown egg within the ovariole.

Fig. 21. Undifferentiated diploid cell from the ovariole.

Fig. 22. Oöcyte nucleus in post-synaptic spireme stage.

Fig. 23. Oöcyte nucleus, later stage in early growth period.

Figs. 24 and 25. Oöcyte nuclei during middle growth period; the chromosomes hazy, diffuse, usually more or less ring-shaped.

Fig. 26. Oöcyte nucleus preparing for the first meiotic division, from Fig. 27.

Fig. 27. Egg, less than 4 minutes after oviposition; the sperm is slightly enlarged and near the center, the oöcyte nucleus near the periphery.

Fig. 28. Egg chromosomes at the end of the first oöcyte division, from Fig. 29.

Fig. 29. Egg, less than 15 minutes old, with first meiotic division completed. The sperm is now swollen and at the center of the anterior part of the egg; the egg chromosomes are near the periphery, those of the polocyte nearest the egg surface.

Fig. 30. First cleavage prophase of a fertilized egg, from Fig. 31; two chromosome groups of five each are distinguishable.

Fig. 31. Egg in prophase of first cleavage; the polocytes are at surface.

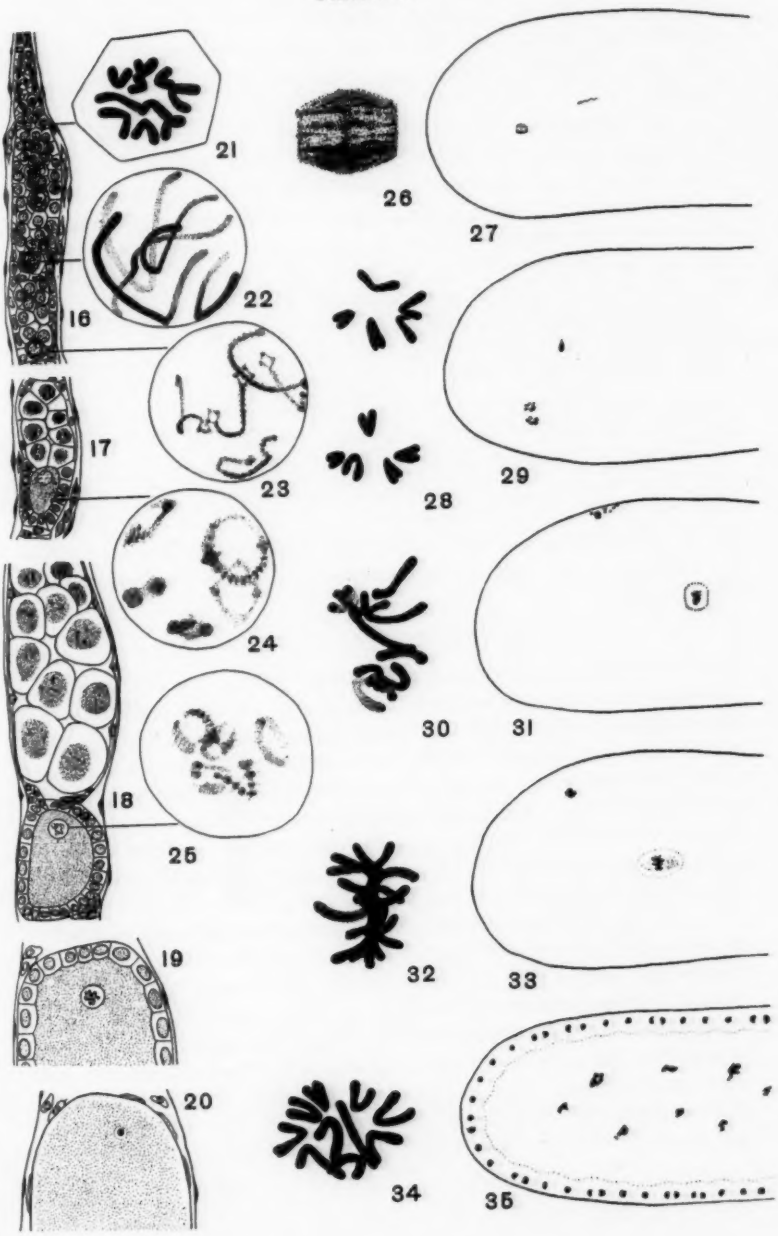
Fig. 32. First cleavage metaphase of a fertilized egg, from Fig. 33.

Fig. 33. Egg in metaphase of first cleavage; the polocytes, optically superposed, are at the surface.

Fig. 34. A metaphase from the blastoderm; from caudal end of egg in Fig. 35.

Fig. 35. Cephalic end of egg with young blastoderm; the yolk nuclei are in the center; the blastoderm cells, in this region, are all in anaphase.

PLATE II



The 5 chromosomes of the second oöcyte and those of the first polocyte are also present and are distinguishable by their relative positions, the oöcyte chromosomes being nearer the center of the egg while those of the polocyte are near the surface. Both are shown on a larger scale in Fig. 11.

After completion of the second oöcyte division, normal, haploid cleavage may set in in a small percentage of the eggs; but it is not at present known whether such normal cleavage can actually occur in eggs containing a foreign sperm or whether it is confined to eggs which may have escaped penetration by a sperm. In any case, whenever cleavage proceeds normally the nuclei are invariably haploid. The first cleavage mitosis occurs in the center of the anterior half of the egg. During the succeeding cleavages, the mitotic stages are strictly synchronous up to the time of formation of the complete superficial blastoderm, 12 hours later, when the divisions at the hind end of the egg begin to lag slightly behind those at the anterior end. Figure 15 represents the anterior end of an egg in which the cells are all in metaphase of the eighth cleavage division. Polar and lateral views of typical cells from this egg are also shown on a larger scale (Figs. 13 and 14), with the slender, elongated chromosomes characteristic of these divisions. In germ-band stages, in late embryos and in young larvæ hatching from such eggs only the haploid number of chromosomes has ever been found.

Development of Fertilized Eggs

In normally fertilized eggs the meiotic stages are like those just described for unfertilized eggs. The sperm is most often found at first caudad to the oöcyte nucleus whence it proceeds to the region where the first cleavage mitosis is to occur. Of the oötids, the one which is to take part in syngamy also approaches this region, while the polocytes come to lie at the periphery of the egg. In an egg less than four minutes old (Fig. 27), the sperm head is slightly swollen and the oöcyte nucleus (shown on a larger scale in Fig. 26), now shaped like a truncated spindle, is also swollen and is approaching the periphery. Figure 29 shows the end of the first meiotic division as well as the enlarged sperm; and Fig. 28, the two chromosome groups more highly magnified. The prophase of the first cleavage mitosis (Figs. 30 and 31) contains 10 chromosomes and it is possible to distinguish two groups of five chromosomes each, one group derived from the male pronucleus, the other from the female. This stage is followed by a metaphase (Figs. 32 and 33), of which only the lateral view is obtainable in an egg which has been mounted entire. The succeeding cleavage divisions and the later stages of development are similar to those

described above for unfertilized eggs, excepting that the diploid number of chromosomes is present. Figure 35 represents an egg in which the cleavage nuclei have just entered the periplasm to form the completed blastoderm. Embedded in the yolk are a number of chromatic masses, the yolk nuclei, while at the surface, each blastoderm cell is in anaphase, excepting those cells at the extreme caudal end of the egg which are still in metaphase. One of these metaphases is shown on a larger scale in Fig. 34.

Discussion

It is apparent that the males in this species are haploid and develop from unfertilized eggs of either mated or virgin females, while the females are always diploid zygotes. Sex determination thus appears to follow the Dzierzon Law except that, in *Melittobia*, haploidy generally has a lethal effect and only a small proportion of the unfertilized eggs is capable of development.

There is nothing, however, in our data that would exclude the validity of the Whiting theory of sex determination. In attempting to apply the latter theory here, we should have to note again that haploidy is lethal in most eggs and that there is as yet no evidence for the survival of zygotes produced by homeosyngamy, i.e., for biparental males.

Biparental males may be looked for only among the offspring of normally mated females. Not more than four such males were examined cytologically. The remainder of the males examined were all obtained from virgins or from females mated with males of a different species. Although the direct evidence against the occurrence of biparental males is inadequate, the fact that the number of eggs yielding males, compared with the total number of eggs laid, is no larger (ca. 3 per cent) in females normally mated than in females not so mated would seem to suggest that biparental males are not produced. We may therefore conclude that homeosyngamy, if it occurs, is lethal. Without the presence of distinguishable X and Y chromosomes the existence of invariably lethal homeosyngamous combinations is not demonstrable. If such combinations do occur, we should expect to encounter a corresponding number of inviable eggs in the layings of normally mated females. This one condition, at least, is actually met. There is normally a considerable egg mortality observed, which seems to vary in different individuals. In the only instance in which an accurate count was made it amounted to 21 per cent out of the first 224 eggs laid.

The fact that the ratio of males to females (3 : 97), produced by

normally mated females, is approximately the same as the ratio of males to unhatchable eggs produced by females not so mated, brings to mind an idea expressed by Lenhossék and Godlewski. As expressed by Godlewski, this idea is that in honey bees two sorts of eggs are produced, of which only the one sort, the female-producing, is capable of and requires fertilization, while the other produces males parthenogenetically. Although it is now generally recognized that in honey bees every egg is capable both of parthenogenesis and of fertilization, the evidence in *Melittobia* appears to favor Lenhossék's idea. However, unless we can state more definitely the nature of the differences between the postulated two sorts of eggs, any hypothesis based on this idea remains rather gratuitous and does not contribute to any real understanding of the mechanism responsible for the unusual sex-ratio and the related reproductive phenomena observed in this species. In the material examined there have not been recognized, as yet, any visible differences in the chromosomal behavior during oögenesis or later, which might be considered responsible for the failure of haploid development in most eggs and its success in some.

LITERATURE CITED

DZIERZON, J., 1845. Ueber die Fortpflanzung der Bienen. Eichstätt. Bienen Zeitung, **1**: 113. (Cited from P. W. Whiting, 1935).

GODLEWSKI, E., 1910–1914. Physiologie der Zeugung. In *Winterstein's Handb. d. vergl. Physiol.*, **3** (2): 457.

LENHOSSÉK, M. v., 1903. Das Problem der geschlechtsbestimmenden Ursachen. Jena. (Cited from E. Godlewski, 1910–1914.)

SCHMIEDER, R. G., 1933. The polymorphic forms of Melittobia chalybii Ashmead and the determining factors involved in their production (Hymenoptera: Chalcidoidea, Eulophidæ). *Biol. Bull.*, **65**: 338.

WHITING, P. W., 1935. Sex determination in bees and wasps. *Jour. Hered.*, **26**: 263.

THE RELATION OF FAT CHANGES TO THE GENERAL CHEMICAL EMBRYOLOGY OF THE SEA URCHIN

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INTRODUCTION

Several years ago (Hayes, 1934) a study was made of the nitrogen metabolism in the early egg stages of the Florida sea urchin, *Echinometra lucunter*. In the previous year Ephrussi published a general account of the metabolism of developing *Paracentrotus* eggs. The papers agreed in finding a loss of nitrogen during later development; for the period immediately following fertilization there are no data in the latter paper, and in the former the results suggested an increase in nitrogen which, since such a state of affairs is otherwise unknown in embryology, was treated with caution pending confirmation. Nitrogen then, and therefore protein, is evidently a source of energy in later development, probably from the eight-hour hatching stage on.

This paper records the results of a study of another possible source of developmental energy, namely fat. Ephrussi (1933) offers the only earlier study, and he found a diminution of fat as development proceeded. Since he tested only three ages, namely unfertilized, 12 hours and 40 hours, his results do not show the precise time at which the loss took place, and cannot be used to supplement and check those to be presented below.

Ephrussi expresses his results as percentages of the wet or dry weight of the egg, a procedure which can be standardized when one is dealing with unfertilized eggs alone, but which presents great difficulties when it is necessary to take into consideration the changes in volume and shape which occur during development. Eggs usually have to be concentrated for analysis by centrifugation, and the number of eggs which will occupy 1 ml. in a centrifuge tube at a given speed is not the same at any two ages. If eggs are sectioned and examined it is possible to derive formulae for the estimation of volume at different stages (Pelluet, 1938). If the volumes are now combined with counts showing how many eggs can be packed into 1 ml., data are at hand for the expression of any egg constituent as a percentage, e.g. fat. This procedure would be very tedious; has not been carried out by any

investigator; and if it were would not be profitable because the truth would be obscured by fluctuations in the water content which would make e.g. a stationary fat concentration per egg appear to vary. For these reasons and others which have been discussed at length (Hayes, 1934), the values below are given per million eggs rather than in per cent.

Methods

The first requirement in work of this kind is a method for rearing eggs in large numbers, free from debris and bacteria, and showing a high percentage of normal development. Attempts were made at first to work with starfish eggs, but the last condition could not be satisfactorily met, and *Arbacia* was substituted. Eggs were obtained from several females, fertilized and reared in finger bowls according to the well-known methods devised by Just and others. All stages were examined to ascertain the percentage developing normally and it was always over 90.

When it was desired to collect a sample the eggs were concentrated in centrifuge tubes at low speeds. This procedure could not be used for swimming stages, which were collected by filtration through fine bolting silk. Following either treatment, eggs or larvae were transferred to a volumetric flask of sea water, usually of one liter capacity, and the water made up to the mark. Water and eggs were then transferred to a larger vessel and thoroughly mixed. While the mixing was continued 1 ml. samples were withdrawn with a brass Stempel pipette and transferred to a Sedgwick-Rafter plankton counting slide of capacity 1 ml., provided with a cover slip. (I am indebted to Dr. C. J. Fish for the loan of a Stempel pipette and counting slide.) Ten fields of the slide, selected at random, were then counted with a microscope whose lenses had previously been calibrated with a stage micrometer. The total was added up and constituted one observation. Usually four such observations were made on each of two samples and the results averaged. Two observers alternated on the counting in order to eliminate the personal factor as far as possible. The probable errors in counting ranged from $\pm$ 0.39 per cent to $\pm$ 10.2 per cent with an average value of $\pm$ 4.1 per cent.

After the counts were made the eggs were again concentrated in centrifuge tubes, and transferred from these to a 100 ml. volumetric flask. The fats were extracted by the wet alcohol-ether method described by Fowweather (1926), the flask was made up to the mark, and the fat-containing extract filtered off and stored in glass-stoppered bottles, evaporation being prevented by sealing the tops of the bottles

with several coats of silica (water glass). When the analyses were made some months later, portions of the alcohol-ether extract were dried in a partial vacuum at room temperature and re-extracted with petroleum ether. These were made up to known volume and stored in cork-stoppered bottles, sealed by silica.

The total fat or phospholipid or sterol digitonide, was estimated by a micro-method devised by Backlin in 1930, described by Peters and Van Slyke in 1932, and slightly modified by Van Slyke, Page and Kirk (1933). Carbon dioxide, produced by the combustion of a fat sample in a special test tube attached to a Van Slyke volumetric or manometric apparatus, is carried over and reacts with dilute NaOH in the chamber, being subsequently liberated by the addition of excess

TABLE I

Variations during development in total fat, sterol and phospholipid per million eggs.

1 Age in hours and minutes	2 Total fat, mg. per million	3 Sterol, mg. per million	4 Phospholipid, mg. per million
Unfert.	5.65	0.430	2.57
1.	3.98	0.413	2.53
4.40	3.50	—	—
6.30	3.36	0.413	3.3
8.50	3.04	—	0.81
10.50	3.60	—	2.04
15.35	3.80	—	2.48
19.30	3.92	0.431	—
23.15	3.84	—	3.46
24.50	4.29	—	—
25.25	3.25	—	1.2
43.10	2.11	0.416	—

lactic acid, and measured as a gas. The probable errors resulting from extractions and fat determinations are less than $\pm$ 2 per cent. For conversion of the CO_2 into fat or into sterol the factors are given in Peters and Van Slyke, page 437. A similar table was constructed for phospholipids, based on data in Bloor (1929).

Sterol was precipitated with digitonin directly from an aliquot of the original alcohol-ether extract in one of the combustion tubes. The precipitate was washed with ether and with water, supernatent fluid being removed by means of suction through an alundum filter stick as described by Kirk, Page and Van Slyke (1934). After drying, the sterol digitonide was oxidized to CO_2 as described above. The method was checked and found to give theoretical values with cholesterol.

Total Fat

By total fat is meant all the material soluble in petroleum ether. Its variations are given in Table I, column 2 and in Fig. 1. It will be noted that from an initial value of 5.65 mg. per million in the unfertilized egg, there is a steady drop until by 9 hours there are only 3.04 mg. From this time until 20–25 hours an increase is noted, followed by a drop to the final stage observed. The most interesting points are that the trough of the curve coincides with the time of hatching, and that there is evidence of fat synthesis from approximately 9 to 23 hours. The shedding of the shell and the beginning of fat synthesis may well be associated with one another. No previous

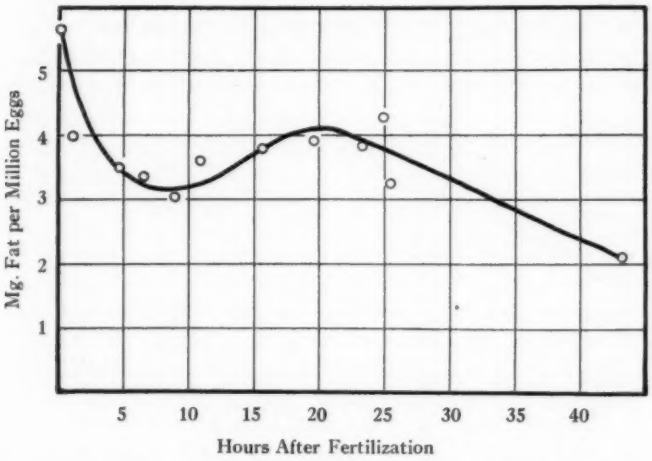

Fig. 1. Fluctuations in total fat during development. Note that the time of hatching, 8 hours, corresponds to the initial trough of the curve.

reference to the manufacture of fat by invertebrates has been found, although the phenomenon is not unknown in teleost embryos (Hayes and Ross, 1936). Since the embryo has at this time no organs of digestion, the source of the new fat is presumably some other material in the egg. There is no proof as to what this is, but a hint is obtained from the nitrogen metabolism of the Florida sea urchin, *Echinometra* (Hayes, 1934), in which, at about the time of hatching a decline in the non-amino nitrogen began, which was practically arrested when the observations closed at 24 hours. (The amino nitrogen, on the other hand, did not begin to decline until the sixteenth hour.)

Ephrussi's three values for total fat were as follows, given in each case as percentage of the wet weight.

Unfertilized	4.81
12 hours	4.43
40 hours	3.69

A glance at Fig. 1 will show that the peak at 20 hours will be missed by the particular stages selected by Ephrussi, so that his gentle decline is in agreement with Fig. 1.

Three further pieces of evidence may be mentioned, after which it will be possible to present a provisional picture of the general chemistry of early development. Warburg (1915) found in *Strongylocentrotus* a sudden burst of respiratory activity at fertilization, followed by a steady increase as development proceeded. The respiratory quotient over the first three hours was 0.9.

Secondly Runnström (1933) observed in *Paracentrotus* a sudden production of acid at fertilization. It was not carbonic acid—in fact the pH of the egg was altered to such an extent that considerable quantities of CO_2 were driven off. The consequence was that Runnström, who was making an investigation of the changes in respiration during the first hour, found that the excess CO_2 caused the apparent R.Q. for the first 10 minutes to soar to 2.37. Within half an hour the mysterious acid was being used up, and respiratory CO_2 consequently held in the egg as bicarbonate, with the result that the apparent R.Q. for 30–45 minutes was only 0.45. Although Runnström did not discover what the acid in question was, he showed that it was not phosphoric acid, and he cited the work of Perlzweig and Barron on *Arbacia* to prove that it was not lactic acid. The inference is that carbohydrate metabolism is not responsible. It may be noted here for future reference that Page (1927) found that the oil of *Arbacia* eggs had a saponification value of 606; of *Asterias* eggs 319. The oils also yielded large quantities of volatile fatty acid when distilled with steam. Fatty acids from such oils would have a low molecular weight, high solubility in water, and adequate strength to drive out CO_2.

Finally, evidence has been accumulating for some years which suggests that two kinds of respiration go on simultaneously in the egg (see Loeb and Wasteneys, 1911). It is not improbable that two substrates are burnt and that two enzymes facilitate the oxidations. That one of these belongs to the general group of oxidases, peroxidases and catalase is shown by the fact that the respiration both of fertilized and unfertilized eggs is stimulated by dimethylparaphenylene diamine (Örström, 1932). This reagent is absorbed by the eggs and becomes

in effect an artificial substrate, the measure of whose oxygen requirement is an index of the available excess enzyme in the egg. The enzyme system then, is present before fertilization, but of course the amount of oxidation which it can bring about is strictly limited by the quantity of available substrate. A second characteristic of the oxidase type of enzyme is its susceptibility to cyanide poisoning. Now cyanide has very little effect on the respiration of the unfertilized egg (Runnström, 1930), but it produces a marked inhibition of oxygen consumption after fertilization. The conclusion is that there is an abundance of enzyme, but practically no substrate before fertilization, and a small but steady supply after fertilization, which is used up as produced. As to the second part of respiration (not inhibited by cyanide) it was found that it could be stopped by narcotics, e.g. lithium (Lindahl, 1934), and stimulated by pyocyanine (Runnström, 1935*a*, both fertilized and unfertilized eggs), and by methylene blue (tried on unfertilized eggs only by Barron, 1929). Thus it passed the tests for a dehydrogenase system. Pyocyanine acts as an artificial enzyme, and the stimulus caused by its presence meant that there must have been some extra substrate there to be burnt up; in other words (in contrast with the first part) the substrate was plentiful and the small quantity of enzyme was the limiting factor.

Taking all these facts into consideration, assuming that the various sea urchins resemble one another in the essential features of their embryonic metabolism, and remembering that the times of hatching approximately coincide, the following general hypothesis of echinoid chemical embryology may be advanced.

(1) At the time of fertilization a considerable quantity of fat is split into fatty acid and glycerol, presumably by means of a lipase, the activation of which is the first of the chain of events initiating embryonic metabolism. It would be tempting to believe that the lipase is brought in by the spermatozoön, but no evidence has been found bearing on this point. For every molecule of glycerol produced there are three molecules of fatty acid; and for every calorie of energy available from glycerol there are 5 or 10 calories available from a lower fatty acid. The glycerol is scarce, the acid plentiful.

(2) The glycerol is rapidly oxidized by a cyanide-sensitive enzyme. This part of respiration does not increase as development proceeds. The enzyme was there before fertilization, but was inactive because of the absence of suitable substrate. The destruction of glycerol accounts in part for the increase of activity found at fertilization by Warburg. Glycerol continues to be produced in small quantities up to at least 8 hours, but is oxidized as formed. That cell division is

the part of development presided over by cyanide-sensitive respiration was shown many years ago by Loeb and Wasteneys (1911), who found that the concentration of cyanide exactly necessary to block development reduced the oxygen consumption to one-quarter its normal value, an amount which coincided with the normal requirement of unfertilized eggs. Runnström (1935*a*) has recently confirmed this experiment, and extended it by immersing eggs in a mixture of cyanide and pyocyanine at the same time, thereby inhibiting one part of respiration and stimulating the other. The net result was an increase in oxygen consumption, but a blocking of cell division in early prophase.

(3) Runnström's unknown acid is fatty acid. It is oxidized by the second, or cyanide-insensitive dehydrogenase system. The activities resulting from this energy source are presumably growth and basal metabolism. The enzyme does not oxidize all its substrate at once—if it did the egg would burn out in a very short time. Nevertheless, this part of respiration is shown by experiment to increase in intensity as development proceeds. An explanation of the paradox of how a limited quantity of enzyme can be made to do more work later in development than at the beginning is obvious from the work of Spek (1934), who showed that in *Asterias* and *Arbacia* eggs acid is not distributed evenly throughout the whole egg, but is strictly localised. Before fertilization the surface is acid, the interior alkaline; in early developmental stages there is a thin acid layer surrounding the dividing cells, and from the 64-cell stage to the gastrula there is a gradient of intensity of acid reaction between the animal and vegetal poles. The chemical geography of the egg limits the extent to which enzyme and substrate are able to come together. This conception is strengthened by Runnström's (1935*b*) statement that "All our experimental evidence indicates that the susceptibility to the action of lithium is highest at the animal pole and decreases gradually."

(4) Fat ceases to be a source of energy at approximately the time of hatching, 8 hours, possibly because it is required as building material.

(5) From hatching time up to 16 hours energy is provided by non-amino nitrogenous materials (non-protein?), from which fat is synthesized and possibly the costs of cell division, motion and basal metabolism are in part or in whole met.

(6) To the utilization of non-amino nitrogenous materials there is added at 16 hours, a gradual destruction of compounds containing amino nitrogen as well.

(7) At 24 hours fat begins once again to decline while at the same time the destruction of nitrogenous material appears to be concluding.

(8) Ephrussi (1933) reports the loss of almost all the carbohydrate in the egg between his 12- and 40-hour tests, and very little loss in the first 12 hours. This suggests that carbohydrate is the fuel source drawn upon in later stages, possibly by about 24 hours. The embryo is perhaps using up its last reserves of food and consequently entering upon a period of starvation, pending the development of its digestive system to a point where food can be taken from the sea.

(9) Hence the apparent sources of energy in succession are: fat, non-animo nitrogen, amino nitrogen, fat, carbohydrate. More detailed information may move carbohydrate to the second or third last place.

The assignment made above of cyanide-sensitive enzyme to glycerol (or a product of it), and of cyanide-insensitive enzyme to fatty acid, is based not only on the relative abundance of the substrates, but also on the work of Emerson (1927), which indicates that cyanide-sensitive respiration results from the oxidation of carbohydrate.

Lindahl and Öhman (1936) have recently placed on some of the results cited above an interpretation differing from that given here. They say, "We see a useful conception of these relationships in the assumption that two different substrates are burnt, of which one has a small, the other a high degree of reactivity to the oxidizing 'carrier.' Shortly after fertilization only the first substrate is present in large quantities. The unchanging and small concentration of the 'carrier' determines the constant and small intensity of oxidation of this substrate. This reaction corresponds to the non-growing part of respiration, which can be increased to a marked degree by dimethylparaphenylene diamine and pyocyanine. The 'carrier' is here the limiting factor. The other substrate is very rapidly oxidized and hence does not accumulate. The rapidity of formation of this substrate determines the oxygen consumption, and is therefore the limiting factor. This is the 'growing part' of respiration, which can be inhibited by lithium." It will be seen that Lindahl and Öhman differ from the views expressed in this paper in that they: (*a*) postulate two substrates but only one enzyme system; (*b*) express no view as to the nature of the substrates; (*c*) consider that dimethylparaphenylene diamine and pyocyanine act on the same part of respiration; (*d*) state that the cyanide-sensitive part of respiration is limited by the low degree of reactivity of the substrate (and not by its scarcity); (*e*) state that the cyanide-insensitive part of respiration is limited by the scarcity of the substrate (and not by geographical segregation).

Sterol

The values for sterol will be found in column 3 of Table I. It amounts to some 0.41–0.43 mg. per million eggs, and no fluctuation was found in its concentration during the period investigated. The only comment necessary is that this particular one-tenth of the fat is not a source of embryonic energy.

Phospholipid

The results of phospholipid determinations are given in column 4. They vary from less than 1 mg. to nearly 3.5 mg. per million eggs. The values unfortunately fluctuate so much that attempts to discover a developmental trend by plotting them on a graph are unconvincing. No utilization of phospholipid can be said to be demonstrated, although the data are not sufficiently extensive to exclude the possibility. The average of all the phospholipid results is 2.17 ± 0.24 mg. per million eggs or 38 ± 4 per cent of the total fat in the unfertilized egg.

Previous Analyses

McClendon (1909) found that the total fat (ether extract) of unfertilized *Arbacia* eggs was 2.254 per cent. Harvey (1932) gives the data necessary to convert this to mg. per million as follows:

$$\text{Mg. per million eggs} = \frac{10^6 \times \text{vol. of 1 egg in cu. mm.} \times \text{egg density} \times 2.254}{100}$$

$$= 2.12 \times 1.09 \times 2.254$$

$$= 5.21 \text{ mg.}$$

McClendon also estimated the phosphorus content of his ether extract and found it to be 0.06914 per cent of the whole egg. Assuming that all the phosphorus is in the phospholipids and that it comprises 4 per cent of these (as it does in lecithin) then this figure too can be made comparable.

$$\frac{2.12 \times 1.09 \times 0.06914 \times 100}{4}$$

$$= 3.84 \text{ mg. phospholipids per million eggs.}$$

If it is valid to apply these calculations to McClendon's results then it follows that they agree in a general way with those given above.

Page (1927) has also made analyses of the fatty constituents of the unfertilized *Arbacia* egg. He states that 8.3 grams of oil were obtained from 183 million eggs by means of alcohol-ether extraction. This is in the terminology of this paper, 45.4 mg. per million eggs, or

ten times as great as McClendon's figures and those given here. A calculation of the mass of an egg from the volume and density values in Harvey shows that, according to Page, 20 per cent of the egg is fat. Now since the total solids (McClendon) only comprise 20 per cent of the egg and these are obviously not all fat, Page's results are difficult to interpret, particularly in view of his statement that *Arbacia* contains much less oil then *Asterias*. Page also precipitated the phospholipid from his extract with acetone, and his results work out at 8.4 mg. per million as against McClendon's 3.84 mg. and the 2.17 mg. above. Finally Page distilled the volatile fatty acids from his extract with steam and titrated them with NaOH, finding 9.4 mg. per million eggs. The discrepancy between Page's results and the others may lie in part in his method of counting eggs, no mention of which is made in his paper. This difficulty would not invalidate his estimates of the saponification value and his volatile acid determinations on which a part of the general argument in this paper depends.

Summary

Periodical estimations were made of the total fat, sterol and phospholipid during the first 40 hours of development of *Arbacia*. The total fat decreases up to the time of hatching (8 hours), then increases for some 10 hours, and later decreases again. The sterol concentration remains unchanged throughout the period studied. Owing to fluctuations in the phospholipid readings, a definite conclusion could not be drawn as to whether this material is utilized as a source of embryonic energy. The total fat in a million unfertilized eggs is 5.65 mg. of which 7.5 per cent is sterol and 38 per cent phospholipid. A general hypothesis of the chemistry of early sea urchin development is presented.

It is a pleasure to acknowledge my indebtedness to Professor E. G. Young, whose advice had been frequently sought and most generously given.

LITERATURE

Backlin, E., 1930. *Biochem. Zeitschr.*, **217**: 482.
Barron, E. S. G., 1929. *Jour. Biol. Chem.*, **81**: 445.
Bloor, W. R., 1929. *Jour. Biol. Chem.*, **82**: 273.
Emerson, R., 1927. *Jour. Gen. Physiol.*, **10**: 469.
Ephrussi, B., 1933. *Arch. de Biol.*, **44**: 1.
Fowweather, F. S., 1926. *Brit. Jour. Exper. Path.*, **7**: 7.
Harvey, E. N., 1932. *Biol. Bull.*, **62**: 141.
Hayes, F. R., 1934. *Carnegie Inst. Wash. Publ.* No. 435 (*Papers from Tortugas Lab.*, **28**: 183).
Hayes, F. R. and D. M. Ross, 1936. *Proc. Roy. Soc., London, Series B*, **121**: 358.
Kirk, E., I. H. Page and D. D. Van Slyke, 1934. *Jour. Biol. Chem.*, **106**: 203.

LINDAHL, P. E., 1934. *Naturwiss.*, **22**: 105.
LINDAHL, P. E. AND L. O. ÖHMAN, 1936. *Naturwiss.*, **24**: 157.
LOEB, J. AND H. WASTENEYS, 1911. *Biochem. Zeitschr.*, **36**: 345.
MCCLENDON, J. F., 1909. *Am. Jour. Physiol.*, **23**: 460.
ÖRSTRÖM, Å., 1932. *Protoplasma*, **15**: 566.
PAGE, I. H., 1927. *Biol. Bull.*, **52**: 164.
PELLUET, D., 1938. *Quart. Jour. Micros. Sci.* In press.
PETERS, J. P. AND D. D. VAN SLYKE, 1932. Quantitative Clinical Chemistry. Williams and Wilkins Co., Baltimore.
RUNNSTRÖM, J., 1930. *Protoplasma*, **10**: 106.
RUNNSTRÖM, J., 1933. *Biochem. Zeitschr.*, **258**: 257.
RUNNSTRÖM, J., 1935*a*. *Biol. Bull.*, **68**: 327.
RUNNSTRÖM, J., 1935*b*. *Biol. Bull.*, **68**: 378.
SPEK, J., 1934. *Protoplasma*, **21**: 561.
VAN SLYKE, D. D., I. H. PAGE AND E. KIRK, 1933. *Jour. Biol. Chem.*, **102**: 635.
WARBURG, O., 1915. *Arch. f. d. ges. Physiol.*, **160**: 324. For a translation of this paper see Warburg, O., 1930. The Metabolism of Tumors. Constable and Co., London.

ON THE SPECIFICITY AND RELATED PROPERTIES OF THE CRUSTACEAN CHROMATOPHOROTROPIC HORMONE

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INTRODUCTION

When the eye-stalks of several brachyuran crustaceans are removed, the animals become pale within two hours (1), (2), (3), (4). Injection of a sea-water extract of the extirpated eye-stalks into the blinded (eye-stalk-amputated) specimens results in the appearance of the dark coloration (3), (5). Although several kinds of chromatophores are involved in these color changes, the movements of the melanophores are chiefly responsible for the resulting external coloration, and in the following experiments were the only chromatophores studied in the fiddler crab, *Uca pugilator*.

RESULTS

Method of Determining the Relative Activity of Eye-stalk Extracts

A new method of assaying the eye-stalk hormone was devised so that determinations could be made within an hour. The techniques of preparing the hormone, injecting the animals, and preparing the animals for the test were essentially the same as described previously (5). The plan of the method was as follows: Each of 8 groups of 15 blinded animals per group was injected with one of 8 different, known concentrations of hormone (1.0 E.S.[1]–0.005 E.S. per cc. of solution). The percentage of animals showing the slightest perceptible response (melanophore stellation) was determined at 5-minute intervals following the time of injection for a period of one hour. If the percentage of animals showing the slightest perceptible response at various concentrations is plotted against time, a series of steep, sigmoid curves is obtained whose inflection points intersect the ordinate at about 50 per cent. The times at which 50 per cent of the animals show the response at different concentrations range from 8 minutes for the strongest to 24 minutes for the weakest. A standard curve (Fig. 1) was then constructed, using a range of concentrations from 0.04

[1] The letters E.S. are the abbreviation for eye-stalk. The letters E.S.H. will be used as the abbreviation of eye-stalk hormone.

E.S./cc.–0.002 E.S./cc. Figure 1 can, therefore, be utilized for determining the relative strength of very dilute solutions of the hormone. For stronger concentrations (1.0 E.S./cc.–0.03 E.S./cc.) Fig. 2 was employed, indicating the relationship between concentration and percentage of animals showing the full response (complete melanophore expansion) at 1 hour following the time of injection. For still stronger solutions, the method previously described (5) was employed. By

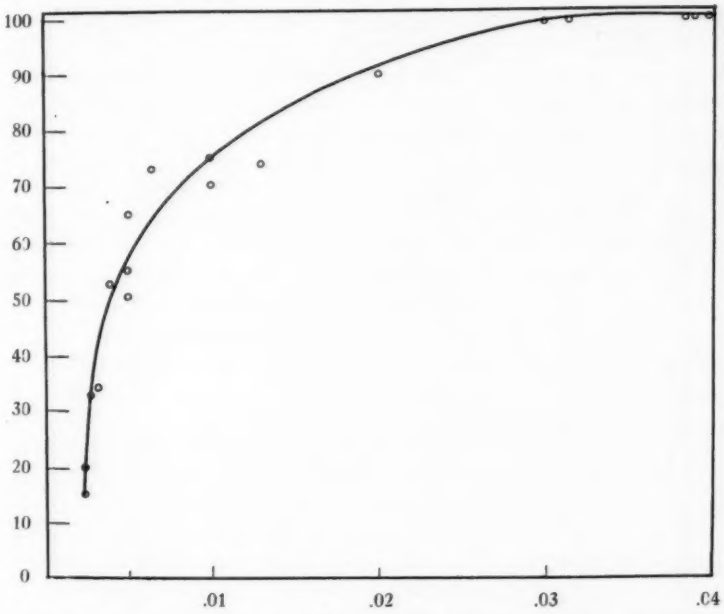

FIG. 1. Curve showing relationship between percentage of animals showing the slightest perceptible response (melanophore stellation) and concentration. Ordinate—percentage of animals responding; abscissa—concentration expressed as eye-stalks per cc. of solution.

using Figs. 1 and 2 as characteristic curves, the relative strength of an unknown concentration can be determined by taking a reading, at 30 minutes after injection, of the percentage of animals showing the slightest perceptible response, and another at 1 hour giving the percentage showing the full response.

A study of these and other curves not included in the text reveals

several points of interest concerning the nature of melanophore response in *Uca*. From the results shown by Fig. 1, where very small amounts of hormone were injected, it is evident that a certain threshold must be reached before the melanophores react and, moreover, that this threshold varies from animal to animal. With stronger doses, the

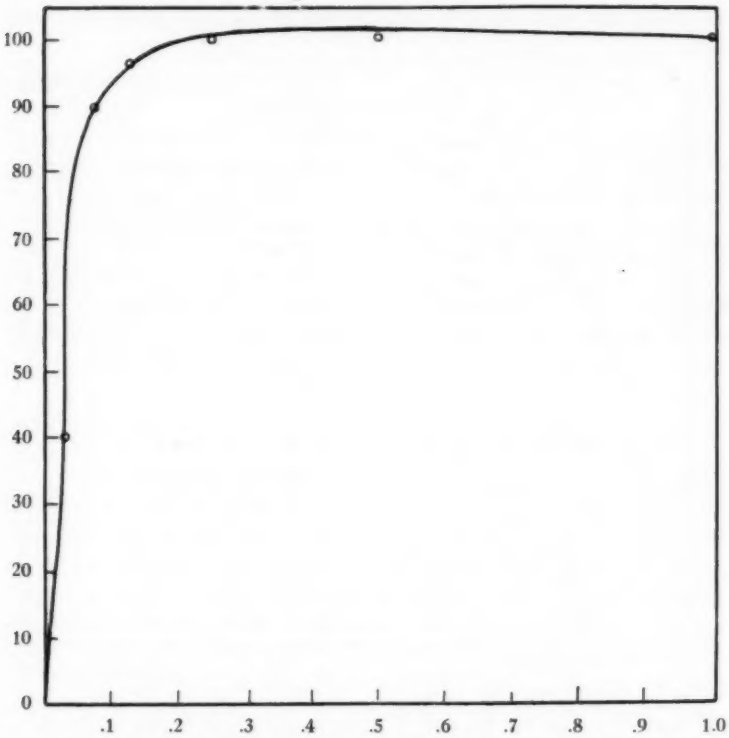

FIG. 2. Curve showing the relationship between percentage of animals showing the full response (complete melanophore expansion) and concentration of hormone. Ordinate—percentage of animals showing full response; abscissa—concentration expressed as eye-stalks per cc. of solution.

degree and the duration of melanophore expansion are exponentially proportional to the dosage injected. Thus, melanophores, like certain types of smooth muscle, show graded responses whose magnitude varies as the strength of the stimulus. For a complete expression among time, concentration of hormone, and the various phases of melano-

phore response, the construction of several nomograms is necessary. The curves already presented, however, suffice for the present studies.

Sensitivity of the Test; Estimation of the Amount of Hormone in the Eye-stalk

The strength of eye-stalk extracts, as read off from the curves, is not constant from animal to animal. Those from larger animals (5.0 grams) usually assay at higher values, although there is always some discrepancy in a group of the same size and sex. The sensitivity of the test may be illustrated by the following experiments: a sea-water extract of 1 eye-stalk (animal weight = 5.0 grams) was diluted until the total volume was 400 cc. Sixty-six per cent of the animals injected with 1/20 cc. of this solution responded. When further diluted to 600 cc., 50 per cent responded; to 800 cc., 33 per cent responded. With still greater dilutions, the percentage quickly fell to zero. We take the point at which 50 per cent of the animals respond as a reliable indication that an extract is active, and in the following discussion, we assume this point (50 per cent response) to indicate a minimal unit of activity, which is 1/600 of a *Uca* unit (5).

The dry or nearly dry weight of an eye-stalk is 2.0 mg. (average of 600 eye-stalks). From microscopic sections of the eye-stalks of *Uca pugilator*, we estimate the sinus gland of Hanström (6) to be, roughly, 1/100 that of the eye-stalk. Assuming the proportion of the active principle to be 1/100 that of the gland, which is probably a conservative surmise, the total amount of hormone in one eye-stalk is therefore about 0.2 γ. The sensitivity of this biological test is such that $0.2 \div 600 \times 20$ (since each animal is injected with 0.05 cc.) or 0.000016 γ of hormone can be detected. These figures, of course, are very rough but, we think, indicate the probable order of magnitude.

Specificity of Action

Thus far, extracts of only two other glands, the pituitary (7) and the subneural gland complex (8), have been found to produce melanophore expansion when injected into blinded *Uca*. We found accidentally that the injection of distilled water produces strong melanophore responses. In 55 crabs, blinded for from 2–8 days previously, the injection of distilled water produced in 15–20 minutes complete melanophore expansion in 90 per cent of the animals. After 1 hour, 50 per cent became pale, and after 2 hours, 90 per cent–100 per cent

became pale. Quantitatively, this effect is quite different from that produced by the eye-stalk hormone in any concentration. Injection of distilled water into 25 normal dark animals was without effect.

Since this reaction is of interest in relation to the general problem of hormone specificity, we examined several possibilities which might explain the effectiveness of distilled water. It is quite certain that injection of sea water does not produce any responses. Only 1 of 46 blinded animals injected with sea water responded weakly but positively, while no significant changes were detected in 10 normal dark animals also injected with sea water. Sea water, therefore, must be employed as the solvent when testing preparations of E.S.H. Since distilled water is acidic (pH 5.7) as compared with sea water (pH 7.7), we injected tap water (pH 7.0) into 10 blinded and 10 normal *Uca*, with results identical to those already described for distilled water. Identical results were also obtained with distilled water made alkaline (pH 8.0) by the addition of 0.01 N NaOH. The acidity of distilled water is therefore not responsible for its chromatophorotropic activity.[2]

Another difference between the action of the hormone and that of distilled water was obtained by injecting various dilutions of sea water with distilled water. The following proportions of sea and distilled water were tried:

Sea Water		Distilled Water	Chromatophorotropic Activity
1 part	plus	1 part	Inactive
1 part	plus	2 parts	60 per cent of animals respond
1 part	plus	3 parts	80 per cent of animals respond
1 part	plus	6 parts	80–100 per cent of animals respond
1 part	plus	10 parts	80–100 per cent of animals respond
1 part	plus	16 parts	80–100 per cent of animals respond

Since the injection of 0.05 cc. distilled water must dilute the blood of a crab appreciably, osmotic effects were studied. Assuming the freezing point depression of *Uca* blood to be close to that of lobster blood, we injected sucrose (1.3 M) isosmotic with the latter into 20 blinded crabs. No melanophore expansion resulted. Sucrose (0.95 M) isosmotic with sea water, however, produced a slight reaction in 20 per cent of the animals. It seemed, therefore, that the effect of distilled water may be due to the resulting hypotonic condition of the blood. The following salts isosmotic with sea water were also tested:

[2] The pH of distilled, tap, and sea water at Woods Hole was determined by means of the glass electrode.

Salt		Blinded Specimens (20 animals per test) General Effects	Chromatophorotropic Activity (per cent of animals responding)
NaCl	0.52 M	—	30
$MgCl_2$	0.51 M	—	20
$CaCl_2$	0.34 M	—	20
KCl	0.53 M	Muscular twitchings and death	0
Na_2HSO_4	0.4 M	Immediate prostration, death after 1 hour	10
LiCl	0.52 M	Immediate twitchings of legs	20
200 per cent sea water		—	0

While the effect of drugs on vertebrate melanophores has been determined, no similar investigation has been made on crustacean melanophores. Sixteen different, chemically-pure drugs were injected into both normal and blinded crabs. One hundred γ of the drugs, dissolved in sea water, was injected into each of 10–20 animals of both groups. Several drugs produced violent, convulsive movements, prostration or twitchings of the legs while others were entirely without muscular effects. Table I summarizes the action of 100 γ of the drugs:

TABLE I

Effects of sixteen drugs on normal and blinded crabs

Drug	Blinded (Pale Animals)		Normal (Dark Animals)	
	General Effect	Chromatophorotropic Activity (Melanophore expansion)	General Effect	Chromatophorotropic Activity (Melanophore contraction)
Atropine sulphate	—	—	—	—
Morphine sulphate	—	—	—	—
Acetyl choline	—	20% (slight)	Shedding of legs	—
Histamine	Paralysis of legs	—	Slight paralysis	—
Eserine sulphate	Prostration and death	—	Not performed	
Pilocarpine HCl	—	—	—	—
Cocaine HCl	Rigidity	30% (slight)	Rigidity	—
Brucine sulphate	Paralysis	—	Sluggishness	—
Veratrine sulphate	Instantaneous death	—	Instantaneous death	50% slightly pale
Curare	Paralysis	50% (slight)	Paralysis	—
Strychnine SO_4	Temporary paralysis	20% (slight)	Paralysis	—
Guanidine	Prostration	20% (slight)	—	—
Chlorbutanol	—	20% (slight)	—	—
Caffein	—	20% (slight)	—	—
Nicotine	Prostration	40% (slight)	Prostration	—
Hyoscine HBr	—	80% positive	—	—

None of these drugs with the exception of hyoscine hydrobromide produced definite, positive results in blinded specimens, and all were without effect on the melanophores in normal dark specimens. These drugs (with the one exception) have therefore no direct or indirect action on contracted or expanded melanophores. We did not repeat these injections in normal animals during the night (nocturnal or pale phase) although possibly some of these drugs may effect a release of the hormone from the sinus gland, which appears to be under nervous control (5).

Adrenalin, practically universally, produces melanophore contraction in vertebrates in extremely small doses. Adrenalin, in various dilutions from 1 : 1,000 to 1 : 10,000 (dilutions greater than 1 : 1,000 being made with sea water) was without significant melanophore responses when injected into either normal or blinded specimens. Strong doses usually produced death in normal animals, while 20 per cent of the blinded animals showed slight melanophore stellation. The activity of adrenalin was confirmed by injecting 0.2 cc. of each dilution subcutaneously in normal dark catfishes. This experiment illustrates effectively the point made elsewhere (4) that melanophores of various animals do not always react in the same way to the same substance, for example, chemically pure adrenalin hydrochloride. It is only logical to think of a response to a hormone in terms of the interaction between the responding tissue and the hormone in question, not solely in terms of the hormone itself. If the same type of responding tissue in two different animals is physiologically and anatomically different, as are the melanophores of crustaceans and vertebrates, it is not surprising that they react in different ways to the same substance. The ineffectiveness of adrenalin in contracting crab melanophores is therefore not a puzzling phenomenon. In fact, it is almost surprising that in one case (7) (4), intermedin produces the same response in crab and vertebrate melanophores.

Do Organs Other than the Eye-stalk Produce the Hormone?

This question has been discussed in detail (5) but as yet no conclusive answer to it has been given. It has been reported (9) that ventral nerve cord extracts of *Palæmonetes* are slightly active on the chromatophores of blinded *Palæmonetes*, and therefore that the ventral nerve cord produces the active principle or principles. A criticism of this conclusion is the possibility that the extracts, prepared from normal animals, may have contained traces of E.S.H. present in the blood bathing the nerve cord.

We have extracted several organs of normal pale, normal dark,

and blinded crabs (blinded previously for various periods of time), and tested the extracts on crabs blinded previously for from 2–8 days. For purposes of comparing the potency of any organ found to be active with that of the eye-stalk, the minimal unit of activity as already described was employed. The wet weight of an eye-stalk is about 6.0 mg., and consequently 600 cc. ÷ 6 mg. or a 0.001 per cent solution of the eye-stalk represents a minimal unit. Stomach, liver, muscle and heart tissue of both normal dark and blinded specimens were extracted by the usual method and prepared in a 0.1 per cent solution; in other words, 100 times more concentrated than that representing a minimal unit of eye-stalk extract. All extracts were found to be completely inactive. Finally, we resorted to extraction of entire, normal dark crabs, immediately after extirpating the eye-stalks. The extract was prepared in a 2 per cent solution, or 2,000 times more concentrated than the minimal unit of eye-stalk extract. When tested, it was found to be 5 times stronger than the minimal unit. Repetition of this experiment with normal pale animals and animals blinded for from 2 days to 1 month gave identical results. We must conclude that either the hormone is present or being produced by some tissue in the body even in the absence of the eye-stalks, or that the positive result is an artifact brought about by the injection of a hypertonic solution. If the former is true, it can be of little or no significance in the normal chromatic physiology of these animals, as shown subsequently.

Behavior of Isolated Leg Melanophores

The behavior of isolated scale melanophores of fish to various ions and organic substances has been studied (10) for a long time. One of the most remarkable results is the behavior of *Fundulus* scale melanophores to sodium and potassium ions, expanding to the former and contracting to the latter. We have studied the melanophores of crabs to various substances by cutting off the legs with a fine scissors at a point near the articulation of the femur with the body, and immersing these isolated legs into various solutions. The results are summarized in Table II, readings being taken every fifteen minutes after immersion in the solutions and continued for 5 hours.

The results are not particularly illuminating. It is significant, however, that the effect of distilled and tap water is similar to that produced when they are injected into the animal. Sea water also acts in the same fashion as when injected into pale animals. The fact that dark legs paled in sea water after 1½ hours may be due to the diffusion, destruction, or inactivation of E.S.H. already present in

the legs. This resembles the last phase of a typical response of blinded crabs to the injection of E.S.H., for when the melanophores begin to contract it requires about 1½ hours to attain complete pallor (5). In view of this, and the slowness of melanophore reaction in crabs, we cannot speak of "ionic effects" on melanophores provided the responses are produced well within an hour. Thus, lithium, sodium, potassium, and calcium chlorides all produce a rapid contraction of expanded melanophores, and consequently, there is no difference, as

TABLE II

Legs of blinded crabs (melanophores contracted)

Solutions		Results of Immersion
$MgCl_2$	.37 M	No change for 4 hours, then expansion of femur melanophores
Sucrose	1.30 M	Same as above, although erythrophores expand within 30 minutes
LiCl	.52 M	No change
Na_2HPO_4	.40 M	Expansion within 15 minutes
NaCl	.52 M	Slight stellation for first hour, then contraction
$CaCl_2$	.34 M	No change for 4 hours, slight expansion on fifth hour
KCl	.53 M	Slight stellation for 1½ hours, then contraction
Sea water		No change during 5 hours
Tap water		Expansion within hour, lasting for 5 hours on femur
Distilled		Same as above

Legs of normal dark crabs (melanophores expanded)

Solutions		Results of Immersion
$MgCl_2$	.37 M	Slight contraction in 15 minutes to 4 hours, then expansion on femur
Sucrose	1.30 M	Slight contraction in ½ hour, expansion after 2 hours
LiCl	.52 M	Contraction in 15 minutes to 5 hours
Na_2HPO_4	.40 M	Remain expanded for 5 hours
NaCl	.52 M	Contraction in 15 minutes to 5 hours
$CaCl_2$	.34 M	Contraction 15 minutes to 4 hours, then expansion on femur
KCl	.53 M	Contraction 15 minutes to 5 hours
Sea water		Contraction after 1½ hours
Tap water		Slight contraction after 1½ hours, but expansion after 4 hours
Distilled		Same as above

found in fish melanophores, between the effects of NaCl and KCl. Melanophores in the legs of blinded animals remain more or less contracted in these solutions for 4 hours, so that the effect of these various cations is the same regardless of the original state of the melanophores. However, since the chloride ion is the common anion to all four salts, the result may well be due to it. We have not pursued this aspect further for it is outside the scope of this paper. Isolated leg melanophores of crabs are not valuable material for such an investigation. Their responses are slow, and usually irregular. Most frequently the

reaction starts at the cut surface of the femur and progresses inwardly towards the tibia. Occasionally, the melanophores of the entire femur respond simultaneously, and rarely do the pigment masses of the tibia react prior to those of the femur.

Is the Pale State Constant in Blinded Animals?

It was stated previously that if tissues other than the eye-stalk are capable of producing E.S.H. they must play an insignificant part, if any, in the normal chromatic physiology of the animals. The basis for this belief is that animals once blinded remain in a pale state as long as they remain alive. In all of the experiments, 15–20 blinded animals were maintained in large crystallizing dishes containing about ½ inch of sea water which was changed daily. Under these conditions, blinded animals have been maintained for 2 months, during which they remained entirely pale.

When blinded animals die, they turn quite dark. Even previous to death, the animals turn gradually dark, and remain dark for some time after death. After observing several hundred blinded animals from day to day, we can state that pallor after enucleation of the eye-stalks is largely constant so long as the crabs remain in a healthy state. Thus far, we have not observed regeneration of the eye-stalk, although several puzzling observations, exceptions to the above statement, have suggested this as a possible explanation. The following protocol illustrates the condition of the melanophores in blinded animals:

Aug. 31—158 animals blinded
Sept. 1—100% pale
Sept. 2—98% pale; 2% dark (dead)
Sept. 4—1% slightly dark; 99% pale
Sept. 5—during day 100% pale; during night 100% pale
Sept. 6–13—animals divided into 4 groups:
white background during day —100% pale
white background during night—100% pale
black background during day —100% pale
black background during night—100% pale
Sept. 13—142 animals remaining:
3% slightly dark
5% fully dark
} = A
92% pale = B
Sept. 14—A = 45% dead; 55% dark; B = 93% pale
Sept. 15—A = 33% dark; 66% pale (day)
100% pale (night)
Sept. 16—A = 84% dark; 16% pale (day)
84% pale; 16% dark (night)

The protocol illustrates that over 90 per cent of the animals blinded for 2 weeks remain continuously pale. From 2 per cent–4 per cent, at the end of two weeks, show a slight diurnal rhythm. Further proof of this behavior was obtained by placing 40 animals, blinded two weeks previously, in a glass dish near a window and 25 similar animals in a glass dish in total darkness.

	Exposed to Normal Day and Night Conditions	Darkness
Sept. 14 P.M.	95% P; 5% slightly D	100% P
Sept. 15 A.M.	95% P; 5% slightly D	100% P
Sept. 15 P.M.	95% P; 5% slightly D	100% P
Sept. 16 A.M.	70% P; 30% slightly D	100% P
Sept. 16 P.M.	97% P; 3% slightly D	100% P
Sept. 17 A.M.	67% P; 33% slightly D	100% P

P = pale. D = dark.

A certain percentage of blinded animals is therefore capable of undergoing periodic changes in color. Usually the extent of melanophore expansion is only slight stellation, yet this is a positive reaction. On examination, it was noticed that the stubs of the eye-stalks in those animals showing diurnal rhythm had healed, and were somewhat elongated. However, the stubs were scraped, cut off, or pulled out of their sockets without altering the slightly dark coloration, and consequently the possibility that the regeneration of the sinus gland had taken place was eliminated. That the slight darkening observed in blinded animals during daylight may be due to the direct action of light is improbable because this phenomenon is limited to only a few animals and occurs only after some time has elapsed following extirpation.

Immersion of Animals in Distilled Water

Fifteen blinded animals were placed in a large dish containing distilled water to a depth of one inch, which was changed daily. After 2 days of this treatment, 9 of the animals became dark (ranging from intermediate to complete darkness) while 6 remained pale. These were segregated, and listed as *A* and *B* groups respectively. On the third day, 4 of the animals in *A* were placed in sea water and designated as *C*, and the 3 groups watched daily:

	A (5 animals)	*B* (6 animals)	*C* (4 animals)
4th day	All dark	1 pale, 5 dark	All pale
5th day	4 dark 1 pale	1 pale, 5 dark	All pale
6th day	3 dark 2 pale	2 pale, 4 dark	All pale

At the end of the week, one-third of all the animals had died, the remaining ten were placed in one dish, containing distilled water. On the eighth day, 6 had become pale, and on the ninth day all had become pale, remaining in this state for 4 more days when all but 2 died. One was now injected with distilled water, which promptly evoked an expansion within 15 minutes, but this response vanished within the next 15 minutes. The other was injected with 0.1 E.S. and responded in 15 minutes, remaining dark for 2 hours.

Distilled water has then the same melanophoric effect when animals are immersed in it as when it is injected into the body spaces. After a week, however, the animals become refractory and the dark coloration is gradually lost, but the animals have not lost the ability to respond to injections of E.S.H. or distilled water.

Chemical Characteristics of E.S.H.

The properties of E.S.H. reported earlier (5) have been confirmed and extended. However, in our experiments, the solubility of E.S.H. in various solvents was determined after the following preliminary treatment: 100 eye-stalks were extracted three times with 10 cc. of distilled water by boiling. The solution was filtered and the filtrate reduced to a volume of 10 cc. under a warm current of air. Two volumes of either pyridine, ethanol or acetone were added, and the solution cooled to 5° C. for one day. A heavy red precipitate forms which is discarded after centrifugation. The supernatant fluid contains practically all of the activity, and after drying, was used as stock material. One extraction with 10 cc. of the following solvents was made of the dry powder equivalent to 10 E.S., and both soluble and insoluble fractions assayed:

Solvent	Percentage of Activity Soluble
	per cent
Absolute ethyl alcohol	45
Absolute methyl alcohol	60
95% ethyl alcohol	60
Absolute acetone	0
Ethyl ether	0
Petroleum ether	1
Benzene	1
Chloroform	0
Ethyl acetate	0
Pyridine	6
95% acetone	2
90% acetone	20
95% methyl alcohol	80

Due to the small amount of material available, we have not concentrated on the purification of the hormone. However, if the stock

material is repeatedly extracted with 95 per cent methyl alcohol, most of the activity can be collected as a soluble fraction, which, on basis of activity per milligram of dry weight, represents a tenfold purification.[3] Aqueous acetone seems to destroy some of the hormone for we have not been able to account for all of the original activity present before fractionation by totalling the activity of the soluble and insoluble fractions.

The hormone is apparently soluble in water throughout the pH range. Stock material (20 E.S.) was easily dissolved in 10 cc. of 0.1 N NaOH as well as in 10 cc. of 0.1 N HCl with the formation of a flocculent, inactive precipitate. If 10 E.S. are dissolved in 10 cc. of 0.1

TABLE III

Diurnal rhythm of *Uca pugilator*

Time of Observation	Normal Day and Night Conditions				Constant Darkness	
	Normal Animals			Animals Blinded 2 Weeks Previously *	Normal Animals	Blinded 2 Weeks Previously
					Indeterminate Background	
	White Background 40 Animals	Black Background 40 Animals	Indeterminate Background 40 Animals	Indeterminate Background 40 Animals	25 Animals	25 Animals
September 13 11:00 A.M.	100% D	100% D	100% D	75% I-P	100% D	4% D
7:30 P.M.	82% P	100% D	100% D	100% P	—	—
12:45 A.M.	100% P	50% I; 50% P	80% P	100% P	50% P; 50% I	100% P
September 14 10:00 A.M.	100% D	100% D	100% P	70% I-P	100% D	100% P
8:00 P.M.	26% P; 74% I	12% P; 88% P-I	80% P	98% P	60% P	100% P
10:00 P.M.	80% P; 20% I	18% P; 82% I-D	100% P	98% P	80% P	100% P
September 15 10:00 A.M.	100% D	100% D	100% D	50% I-D	100% D	100% P
4:30 P.M.	100% D	98% D	100% D	87% P	25% P	100% P
10:30 P.M.	90% P	50% P	66% P	87% P	92% P	100% P
September 16 11:45 A.M.	100% D	100% D	100% D	40% I	96% D	100% P
10:30 P.M.	100% P	25% P	100% P	90% P	84% P	100% P
September 17 10:30 A.M.	100% D	100% D	100% D	40% I	84% D	100% P

[3] It was reported (8) that the hormone was precipitated from 95 per cent acetone by the addition of ether. This is an error since in this case the hormone was precipitated from 95 per cent methanol, not 95 per cent acetone.

TABLE III—*Continued*

Time of Observation	All Animals Now Exposed to Constant Light (75 Watt Electric Bulb)					
	Normal Animals			Animals Blinded 2 Weeks Previously *	Normal Animals	Blinded 2 Weeks Previously
					Indeterminate Background	
	White Background 40 Animals	Black Background 40 Animals	Indeterminate Background 40 Animals	Indeterminate Background 40 Animals	25 Animals	25 Animals
September 18 11:15 A.M.	100% D	100% D	100% D	40% I	100% D	55% I-P
3:00 P.M.	98% D	100% D	100% D	25% I	100% D	50% I-P
10:15 P.M.	40% P; 60% I-D	14% P	33% P	3% I; 97% P	33% P	100% P
September 19 12:30 P.M.	100% D	100% D	100% D	25% I	100% D	25% I-P
10:00 P.M.	30% P	20% P	50% P	92% P	50% P	100% P
September 20 12:30 P.M.	100% D	100% D	100% D	12% I	100% D	25% I-P
11:00 P.M.	75% P	33% P	75% P	92% P	50% P	75% P
September 21 12:30 P.M.	100% D	100% D	100% D	14% I	100% D	50% I
10:30 P.M.	50% P	20% P	50% P	90% P	33% P	90% P
September 22 12:40 P.M.	100% D	100% D	100% D	10% I	100% D	42% I
10:20 P.M.	50% P	10% P	60% P	94% P	50% P	90% P
September 23 11:10 P.M.	50% P	6% P	33% P	91% P	33% P	100% P
September 25 1:00 A.M.	50% P	14% P	80% P	96% P	12% P	75% P

P = pale.
D = dark.
I = intermediate.
I–D = intermediate to dark.
I–P = intermediate to pale.

* These were chosen from several hundred specimens because they were not entirely pale.

NaOH and, after 3 minutes, neutralized to pH 7.0 and tested, complete activity is found. If a similar solution is placed at 100° C. for ten minutes, total loss of activity occurs. Ten E.S. in 10 cc. of 0.1 N HCl placed at 100° C. for ½ hour, neutralized, and tested, retains its original activity. In fact, HCl seems to potentiate the hormone somewhat, although we have not studied this carefully. Attempts to regenerate the activity of stock material treated for 15 minutes with NaOH by boiling it with an equivalent amount of HCl after neutralization were unsuccessful.

The Diurnal Rhythm of Uca pugilator

It has been stated (5) that the diurnal chromatic rhythm of *Uca pugilator* proceeded under constant illumination or darkness, and regardless of background. This statement was based upon observations made on only the melanophores of the abdominal segments, since the ventral surface of these crabs can be most easily observed. Since all the experiments reported here deal with the reactions of leg melanophores, we studied for 2 weeks the behavior of the leg melanophores during the periodic change in coloration. Essentially, the same situation as previously described was found, but several new facts of interest were discovered. The following protocol summarizes the observations upon 170 specimens kept under various conditions:

It will be seen that when normal animals are exposed to normal day and night environment, the diurnal rhythm proceeds as described previously on both white and indeterminate backgrounds. On black background, however, the appearance of the nocturnal hue is delayed and does not occur in a certain percentage of animals. Under constant light, the same situation is true except that fewer animals on all backgrounds show the nocturnal phase. Animals maintained in darkness show the nocturnal coloration at night but when exposed to constant light, the number of specimens becoming pale at night is decreased. Two new facts therefore emerge: (1) that black background delays and inhibits the pale phase more effectively under conditions of constant illumination than under normal condition daylight and darkness, (2) that constant light does the same regardless of background.

Observations on the ventral surface of these specimens were made simultaneously but excluded from the protocol. If readings were based upon the behavior of the abdominal melanophores alone, the protocol would indicate that the pale phase occurred in practically all of the specimens regardless of background or of conditions of illumination. There is, therefore, a decided difference between the reactions of the leg and abdominal melanophores.

The diurnal rhythm of *Uca* has been explained on the assumptions that during the day, E.S.H. is released into the blood stream and that during night, release is stopped. On this basis, it must be assumed that the abdominal melanophores are more sensitive than those of the legs to the disappearance of the hormone from the blood stream. In addition to the circumstantial evidence obtained by watching single animals or groups of animals during the diurnal rhythm, further proof was obtained by removing the eye-stalks of 15 dark animals during the day. After 1 hour, 20 per cent of the animals showed white

abdomens but the legs remained dark in all cases. After 1½ hours, 66 per cent of the specimens showed white abdomens and 20 per cent, pale legs. After 2½ hours, all showed pale abdomens while 70 per cent showed pale legs. This experiment, therefore, indicates quite clearly that the abdominal melanophores respond more rapidly than leg melanophores to the loss of E.S.H. from the blood, as brought about by removal of the eye-stalks.

The assumption that the pale phase of *Uca* is due to the absence of E.S.H. from the circulation during the night is based on no direct evidence inasmuch as we have not succeeded in obtaining the blood of *Uca* for assaying E.S.H. The evidence for this belief is, therefore, indirect, and is based on one fact—that pallor in *Uca* is always associated with and due to the loss of the eye-stalks, and hence removal of the chief if not the only significant source of E.S.H. However, it may be possible that the melanophores become refractory during the night to the eye-stalk hormone (which may be thought of as being secreted constantly) thus ushering in the pale phase, or that the pale phase is due to a melanophore-contracting hormone, concentrating nerves, or some unknown factor. The first possibility was negated by injecting some E.S.H. into 20 normal animals showing the nocturnal phase. All responded normally by darkening. The second possibility has no experimental basis inasmuch as no extract of *Uca* has been prepared which induces pallor, and as chromatophore nerves have not been demonstrated in the Crustacea.

Consequently, the explanation of the diurnal rhythm of *Uca* on the basis of the presence and absence in the blood stream of one hormone (E.S.H.) seems to be the most economical, and in conflict with no observational and experimental facts. The effect of constant light and of black background can therefore be thought of as prolonging the release of the eye-stalk hormone into the circulation, or as inhibiting or delaying the process by which the release of the hormone from the gland is stopped.

Discussion

Since most of the data have already been analyzed, we would limit this to a short theoretical discussion on the nature of action of the eye-stalk hormone. It is evident that the problem of the endocrine control of chromatophores in crustaceans is not as simple as has been regarded. While in general the comparative endocrinology of melanophore responses in both vertebrates and invertebrates is rather well understood, we have no inkling of the microphysiology of either the melanophores or the chromatophorotropic hormones in action. In

fact, there is nothing known, with one or two exceptions, concerning the intimate mechanism by which a hormone is able to affect a tissue. The finding that distilled water expands the melanophores of blinded crabs may furnish a clue as to the manner in which the eye-stalk hormone acts, even though the reactions may not be comparable. Since the characteristic action of E.S.H. is the induction of protoplasmic movements in the melanophores, one is inclined to suspect the ionic or osmotic environment as the regulator of melanophore movements. This may or may not be reasonable but, at least, the effect of distilled water can be attributed to the resulting hypotonicity of the blood which in turn might cause a rapid shift in the osmotic equilibrium between the blood and tissues. The expansion of the melanophores might then result from such a change in the osmotic or ionic environment.

There are two possibilities concerning the way in which E.S.H. acts on the melanophores—directly or indirectly. Discussing the second possibility first, it is quite possible that the eye-stalk hormone may not have any relation to changeable coloration at all, but that it regulates the salt or water balance of the animal. The effect on melanophores may well be indirect, resulting from osmotic or ionic changes between the blood and tissues when the hormone is normally present and absent in the blood stream. This would mean, of course, that the chromatophorotropic hormone is only secondarily chromatophorotropic; its main function being in regulating some other process. Indeed, it has been maintained (11) that since this hormone is found in crustaceans which do not show color changes or even contain chromatophores, it must perform some function more important than that of regulating color changes. Such an argument is helpful for the above discussion, but we feel that the presence of a particular hormone may not necessarily mean that it must be performing some function. For example, intermedin is abundantly present in the pituitary glands of both birds and mammals, yet its presence is not correlated with any functional significance. Similarly, the presence of estrogenic hormones in the mammalian testes does not seem to be correlated with any of the normal processes in the reproductive physiology of the male. There are other instances of this kind, but of course some function for the hormones mentioned may be discovered eventually.

To the effect that E.S.H. acts directly on the melanophores, we might add that all of our curves relating response to concentration and time are of a type which has been assumed to represent reversible compound formation on cell surfaces—but this is all that we can marshal in support of a direct action. Isolated leg melanophores will expand when immersed into a solution of sea water containing E.S.H.,

but this is of course no indication of a direct action. Similarly, the effectiveness of various salts on isolated melanophores offers nothing to indicate that the action of E.S.H. is direct or indirect. The determination of the mechanism of action of the eye-stalk hormone belongs properly to general physiology and is perhaps one of the most significant aspects of melanophore physiology. It is hoped that some of these observations will furnish a basis for further investigation.

In concluding, it can be mentioned that a search for other endocrine functions of the eye-stalk such as have already been found (regulation of heart (12), calcium metabolism (13)) will be of importance in establishing the proper rôle which the eye-stalks play in regulating the pigmentary effectors. The hormone is not indispensible to the life of crustaceans. Blinded crabs molt frequently (perhaps more frequently than normal specimens according to our impression) and if well fed can be maintained in a healthy state for several months, and perhaps indefinitely.

Summary

1. A method for determining within an hour the relative activity of eye-stalk extracts of *Uca pugilator* has been described.

2. The amount of hormone present in one eye-stalk of *Uca* (5.0 grams) was calculated to be 0.2 γ. The minimal amount of hormone detected by the method described was calculated to be 0.000016 γ, which represents a minimal unit of eye-stalk activity.

3. Distilled water is effective in expanding melanophores when injected into blinded specimens. The effect seems to be related to osmotic changes induced in the animal. Excepting the effect of distilled and tap water, the response of blinded animals to the eye-stalk hormone seems to be quite specific. Sixteen different drugs were ineffective in causing melanophore expansion.

4. Blinded animals immersed in distilled water become and remain dark for a week, after which they become refractory. However, they have not lost the ability to respond to injections of either eye-stalk hormone or distilled water.

5. Extracts of several organs other than the eye-stalk were found to be inactive on blinded specimens, even when 100 times more concentrated than a minimal unit of eye-stalk extract. Extracts of entire bodies, 2,000 times more concentrated than a minimal unit, were active.

6. Isolated leg melanophores respond slowly and irregularly to various ions. Na, K, Ca, Li chlorides all induce melanophore contraction. Na and K do not produce opposed responses in isolated leg melanophores as they do in isolated scale melanophores.

7. The relative solubility of the eye-stalk hormone in various organic solvents was determined. The hormone is inactivated by alkali, but the activity cannot be regenerated by treatment with acid.

8. Animals blinded for 2 weeks or more remain continuously pale, with the exception of 2–4 per cent of the specimens, which show slight periodic changes in coloration.

9. The diurnal rhythm of *Uca* is affected by constant light, and black background, both of which delay the appearance of the nocturnal hue on the legs.

LITERATURE CITED

1. MEGAŠUR, F., 1912. *Arch. f. Entw.-mech.*, **33**: 462.
2. ABRAMOWITZ, A. A., 1935. *Proc. Nat. Acad. Sci.*, **21**: 677.
3. CARLSON, S. P., 1935. *Proc. Nat. Acad. Sci.*, **21**: 549.
4. ABRAMOWITZ, A. A., 1938. *Physiol. Zoöl.* (in press).
5. ABRAMOWITZ, A. A., 1937. *Biol. Bull.*, **72**: 344.
6. HANSTRÖM, B., 1931. *Zeitschr. f. Morph. u. Ökol. d. Tiere*, **23**: 80.
7. ABRAMOWITZ, A. A., 1936. *Proc. Nat. Acad. Sci.*, **22**: 521.
8. ABRAMOWITZ, A. A., 1936. *Anat. Rec.* (Supplement No. 1) (Abstract) **67**: 108.
9. BROWN, F. A., JR., 1935. *Jour. Exper. Zoöl.*, **71**: 1.
10. SPAETH, R. A., 1913. *Jour. Exper. Zoöl.*, **15**: 527.
11. HANSTRÖM, B., 1937. *Kungl. Svenska Veten. Hand.*, **16**: 3.
12. WELSH, J. H., 1937. *Proc. Nat. Acad. Sci.*, **23**: 458.
13. KOLLER, G., 1930. *Zeitschr. vergl. Physiol.*, **12**: 632.

THE EFFECT OF SODIUM, POTASSIUM AND CALCIUM IONS ON CHANGES IN VOLUME OF AMOEBA PROTEUS[1]

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INTRODUCTION

McCutcheon and Lucké (1928) found that, with the same osmotic concentration, permeability of the eggs of *Arbacia* to water is greater in solutions of dextrose containing 0.01 M NaCl or KCl and less in solutions of dextrose containing 0.004 M $CaCl_2$ or $MgCl_2$ than in solutions of dextrose in distilled water. These results indicate that sodium and potassium ions increase and calcium and magnesium ions decrease permeability to water. Jacobs (1930) showed that hemolysis of erythrocytes in solutions of non-electrolytes, 0.01 M or 0.02 M, is almost as rapid as it is in distilled water; but that in solutions of electrolytes, e.g. NaCl 0.001 M or $CaCl_2$ 0.0001 M, it is considerably slower. These results indicate that sodium as well as calcium ions decrease permeability to water, i.e. that under some conditions monovalent as well as divalent salts cause decrease in permeability.

The experiments considered in the following pages were undertaken to ascertain the effect of sodium, potassium and calcium ions in phosphate buffer solutions (0.002 M, pH 6.8) on changes in the volume of *Amoeba proteus*.

MATERIALS

Amoeba proteus was used exclusively in these experiments. It was grown in cultures prepared according to the method described in an earlier paper (Mast and Fowler, 1935). The distilled water used was triple redistilled from a tandem glass still (Mast, 1928). The solutions used were prepared by making two stocks, one containing the primary phosphate of the cation to be tested, 0.002 M, and the other containing the hydroxide of the same cation, 0.002 M, and mixing the two stocks

[1] This paper is the second in a series on the relation between environmental factors and water-content in *Amoeba proteus*. The first paper in the series, entitled "Permeability of Amoeba proteus to Water," was published in the *Journal of Cellular and Comparative Physiology*, Vol. 6, pp. 151–167, 1935.

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in the proportions necessary to maintain the hydrogen ion concentration at pH 6.8. The preparation and standardization of the hydroxide stock solutions is described in the paper cited above. All the solutions thus prepared contained the same concentration of the metallic cation used and the osmotic concentration of these solutions was practically the same as that in which the amoebae were grown, i.e. modified Ringer solution.

If the salts in the solutions used had been completely ionized, the osmotic concentration of the solutions containing calcium would have been greater than those containing either sodium or potassium because if $CaH_4(PO_4)_2$ ionizes there are produced two phosphate ions for each

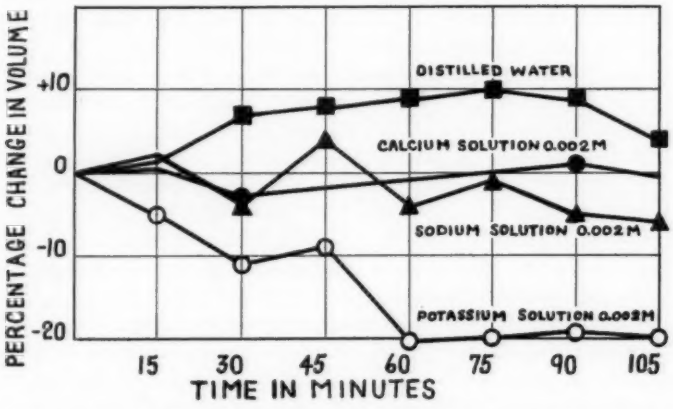

FIG. 1. Graphs showing the relation between sodium, potassium and calcium respectively in phosphate buffer solutions (pH 6.8) and changes in volume in *Amoeba proteus* transferred from dilute Ringer solution of the same osmotic concentration. Each point on the curves represents the average of the results of three measurements made on three different individuals. Ordinates, change in volume as percentage of the original volume; abscissæ, time in minutes after transfer from dilute Ringer solution.

calcium ion, whereas if KH_2PO_4 or NaH_2PO_4 ionizes there is produced only one phosphate ion for each potassium or sodium ion. At the concentration used, however, the sodium and the potassium compounds were slightly more ionized than the calcium compounds. The number of ions per unit volume and the osmotic pressure were probably therefore practically the same in the solutions used.

METHODS AND RESULTS

Three methods were used in ascertaining the effect of these solutions on changes in volume of *Amoeba*.

1. Six amoebae were prepared, selected, and measured individually in modified Ringer solution as described in the paper cited above (Mast and Fowler, 1935). Then they were transferred to distilled water and measured at fifteen-minute intervals. Then this was repeated with other individuals, using in place of distilled water phosphate buffer solutions which contained sodium, potassium and calcium respectively, but with only three individuals in each. The results obtained are presented in Fig. 1.

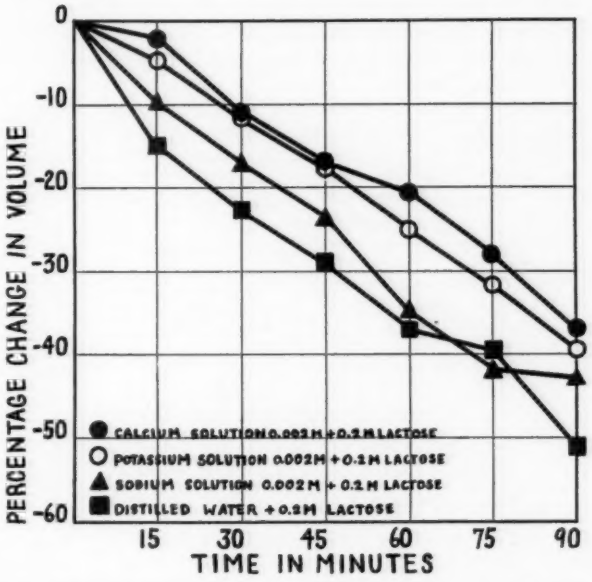

FIG. 2. Graphs showing the relation between sodium, potassium and calcium respectively in hypertonic solutions of lactose (pH 6.8) and changes in volume in *Amoeba proteus* transferred from dilute Ringer solution.

Each point on the curves represents the average of the results of ten measurements made on 7 to 12 different individuals. Ordinates, change in volume as percentage of the original volume; abscissæ, time in minutes after transfer.

Figure 1 indicates that the amoebae transferred from modified Ringer solution to distilled water increased 10 per cent in volume in 75 minutes; that those transferred to the calcium phosphate buffer solutions of practically the same osmotic concentration as the Ringer solution, did not change appreciably in volume during the time represented in the graph, and that those transferred to the sodium phosphate buffer solution decreased only slightly if at all during this time, but that those transferred to the potassium phosphate buffer

solution of the same osmotic concentration as the preceding solutions, decreased 20 per cent in 60 minutes and then remained constant.

After the experiment had continued 105 minutes, the amoebae in the distilled water and in the calcium phosphate buffer solution were normal and they continued to live for several days; those in the sodium phosphate buffer solution soon began to round up and decrease in volume and they disintegrated about 60 minutes later; and those in the potassium buffer solutions disintegrated even sooner. However, those in all the solutions recovered fully in culture solution, if they had not been in the salt solutions more than one hour. The results obtained show, therefore, that without any change in osmotic concentration potassium causes rapid loss of water in *Amoeba*, without any appreciable injury; and that sodium and calcium have little if any effect.

TABLE I

Percentage changes in volume in amoebae transferred from modified Ringer solution (0.002 M, pH 6.8) to a solution containing sodium as the only metallic cation, of the same hydrogen ion and osmotic concentration as the Ringer solution, plus 0.2 M lactose. Temperature, 22.7°–25.5°; E_m, probable error.

Designation of specimen	Time in minutes after transfer						
	15	30	45	60	75	90	105
1	− 7	−17	−17	−27	−32	−32	−46
2	− 2	−18	−37	−48	−55	−60	−55
3	−13	−20	−25	−29	−34	−46	−46
4	−14	−23	−26	−40	−53	−52	−62
5	− 8	−24	−45	−61	−63		
6	−17	−20	−28	−26	−33	−35	−43
7	+ 1	− 9	−21	−31	−36	−41	−44
8	−16	− 9	− 7	−41	−36	−27	−27
9	−18	−26	−23	−34	−41	−39	−51
10	− 3	− 5	− 8	−16	−41	−58	
Mean	−10	−17	−23	−35	−42	−43	−47
E_m	±1.382	±1.442	±2.36	±2.58	±2.18	±2.44	±2.28

2. Several amoebae were prepared, selected, and measured as in method 1, and then transferred to 0.2 M lactose in distilled water, and measured at fifteen-minute intervals. Then this was repeated with phosphate buffer solutions which contained lactose (0.2 M) and sodium, potassium and calcium (0.002 M) respectively. The results obtained are presented in Fig. 2 and Tables I, II, and III.

Figure 2 indicates that the amoebae which were transferred from modified Ringer solution to hypertonic solutions, consisting of lactose

in distilled water and respectively sodium, potassium and calcium plus lactose, decreased in volume in all the solutions, but that the decrease was least in the solution containing calcium and greatest in distilled water plus lactose.

Tables I, II, and III show that there was marked individual variation in the reduction in the volume of the amoebae in all the hypertonic solutions tested, but that in spite of this variation, the probable error is relatively so small that there is overlapping at only a few points on the curves. This practically proves that the differences in the means of the reduction in volume in the different solutions, indicated in the

TABLE II

Percentage changes in volume in amoebae transferred from modified Ringer solutions (0.002 M, pH 6.8) to a solution containing potassium as the only metallic cation, of the same hydrogen ion and osmotic concentration as the Ringer solution, plus 0.2 M lactose. Temperature, 22.1°–25.2°; E_m, probable error.

Designation of specimen	Time in minutes after transfer						
	15	30	45	60	75	90	105
1	− 2	− 8	−19	−23	−28	−23	−36
2	−10	−21	−24	−30	−28	−35	−33
3	+10	−11	−16	−23	−35	−43	
4	− 2	−10	−15	−15	−28	−20	−34
5	−21	−14	−22	−31	−24	−43	−43
6	−11	−17	−23	−32	−42	−57	
7	−11	−14	−11	−30	−37	−43	
8	+10	− 4					
9	− 4	− 4	−11	−29	−37		
10	−17	−17	−17	−20	−20	−46	
11	− 9	− 9	−16	−20	−34	−43	
12	−11	−11	−18	−24	−32	−42	−58
Mean	− 6	−12	−18	−25	−32	−40	−41
E_m	±1.79	±0.97	±0.85	±1.07	±1.26	±2.10	±2.80

curves in Fig. 2, are significant and consequently that the order of effectiveness on preventing loss of water in *Amoeba*, is Ca>K>Na, with very little difference between calcium and potassium.

The facts that the osmotic concentrations of the solutions which contained respectively sodium, potassium and calcium plus lactose were practically equal and that the osmotic concentration of these solutions was somewhat higher than that of the distilled water plus lactose, show that the monovalent sodium and potassium ions as well as the divalent calcium ions, retard the loss of water, but that calcium ions are more effective than sodium or potassium ions.

3. Ten amoebae were prepared, selected, and measured in modified Ringer solution as in methods 1 and 2, then they were transferred to this solution containing lactose (0.2 M), left 30 minutes and measured again. They were then transferred to distilled water and measured at 30-minute intervals. This whole process was then repeated with other specimens in respectively sodium, potassium and calcium phosphate buffer solutions (0.002 M) in place of distilled water. The results obtained are presented in Fig. 3.

Figure 3 shows that the amoebae which were transferred from modified Ringer solution to modified Ringer solution containing lactose (0.2 M) decreased in volume to about 70 per cent of the original volume in 30 minutes and that after they had been transferred from this solution to distilled water or to phosphate buffer solutions containing

TABLE III

Percentage changes in volume in amoebae transferred from modified Ringer solution (0.002 M, pH 6.8) to a solution containing calcium as the only metallic cation, of the same hydrogen ion and osmotic concentration as the Ringer solution, plus 0.2 M lactose. Temperature, 22.5°–24.0°; E_m, probable error.

Designation of specimen	Time in minutes after transfer						
	15	30	45	60	75	90	105
1	− 8	− 2	−11	−20	−23	−29	
2	−12	−22	−25	−17	−29	−44	−53
3	−12	−21	−23	−26	−41		
4	+ 7	− 8	− 6	−13	−30	−36	−43
5	+ 4	− 5	− 8				
6	+ 7	−18	−27	−29	−32	−38	−47
7	− 3	− 5	−21	−17	−13	−42	
Mean	− 2	−12	−17	−21	−28	−38	−48
E_m	±2.01	±1.98	±2.01	±1.53	±1.93	±1.55	±1.61

sodium, potassium or calcium, they increased in volume, but that the increase was much more rapid and much greater in distilled water than in any of the salt solutions. The figure shows that in all the salt solutions the amoebae increased to a maximum and then decreased, but that increase was greater in the solution which contained sodium than those which contained potassium or calcium, the maximum increase being 22 per cent for sodium and only 9 per cent for potassium and 4 per cent for calcium. It also shows that in distilled water the amoebae nearly regained their original volume in 30 minutes, and then continued to increase as long as measurements were made, i.e. for 90 minutes, and that at the close of the experiment they were 17 per cent larger than at the beginning.

The individual variations in the increase in volume in the hypotonic solutions were essentially the same as those recorded in Tables I, II and III for hypertonic solutions, but the differences in the means were greater so that there was no overlapping of the probable errors, except at the last points on the curves. The differences in the means of the increase in volume in the different solutions indicated in the curves in Fig. 3, are therefore significant, showing that the order of

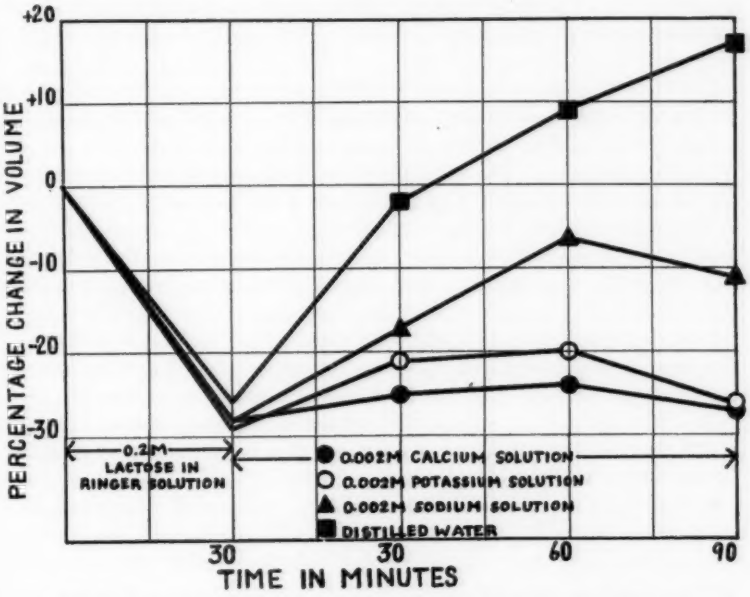

FIG. 3. Graphs showing the relation between sodium, potassium and calcium respectively in hypotonic buffer solutions (pH 6.8) and changes in volume in *Amoeba proteus* transferred from weak Ringer solution containing 0.2 M lactose.

Each point on the curves represents the average of the results obtained in ten measurements made on ten different individuals. Ordinates, change in volume as percentage of the original volume; abscissæ, time in minutes after transfer.

effectiveness on gain of water in *Amoeba* in hypotonic solutions is, Ca>K>Na, that is, that it is the same as it is on loss of water in hypertonic solutions.

By comparing Fig. 3 with Fig. 1, it will be seen that if *Amoeba* is transferred from Ringer solution (0.002 M) to potassium phosphate solution (0.002 M) it decreases markedly in volume, but that if it is transferred from lactose solution (0.2 M) to potassium phosphate

solution (0.002 M) it increases somewhat. This difference is doubtless correlated with the fact that the water-content was much higher in the amoebae which were transferred from the modified Ringer solution than it was in those which were transferred from the lactose solution.

The results presented in Fig. 3 show therefore that monovalent as well as divalent cations retard the entrance of water but that, just as in reference to loss of water, the latter are more effective than the former; and they support the conclusion reached above that both monovalent and divalent metallic cations tend to maintain the water-content in *Amoeba* constant.

Discussion

The question now arises as to how the metallic ions act in their tendency to maintain the water-content of *Amoeba* constant.

Under normal conditions much water is eliminated by the contractile vacuole and considerable water enters with the food in the process of feeding (Mast and Hahnert, 1935) and some possibly enters in the formation of cups without food (Mast and Doyle, 1934). It may be then that the metallic ions influence the water-content of *Amoeba* through action on some or all of these processes. Such action could, however, not have been seriously involved in the results presented in the preceding pages; for in the solutions used there was, during the time the observations were made, no feeding and no formation of cups of any sort and the contractile vacuole did not function except in a few of the more dilute solutions and then only after the amoebae had been in the solution half an hour or more. The changes in water-content observed must therefore have been due to passage of water directly through the surface layer of the amoebae, and the effects of the metallic ions on the rate of the passage through this layer must have been due either to their action on the permeability of this layer or to their action on the osmotic concentration of substances in the amoebae, or to their action on imbibition, but it is probably largely due to the first. This matter will be discussed more fully in a subsequent paper.

The conclusion that monovalent as well as divalent metallic cations tend to maintain the water-content of *Amoeba* constant, is in agreement with the results obtained by Jacobs, who showed that in erythrocytes permeability to water is decreased by the addition of either NaCl or $CaCl_2$ to distilled water; but they are not in agreement with those obtained by McCutcheon and Lucké, who showed that permeability of the eggs of *Arbacia* to water is increased if NaCl or KCl is added to a solution of dextrose in distilled water and decreased if $CaCl_2$ or $MgCl_2$ is added.

Summary

1. If amoebae are transferred from modified Ringer solution (0.002 M, pH 6.8) to distilled water or to phosphate buffer solution containing respectively sodium, potassium and calcium as the only metallic cation (0.002 M, pH 6.8), they increase in size in distilled water, decrease in size in the solution which contains potassium and remain nearly the same in size in the solution which contains sodium or calcium.

2. If they are transferred from modified Ringer solution (0.002 M, pH 6.8) to distilled water containing lactose (0.2 M) or to phosphate buffer solutions containing lactose (0.2 M) and respectively sodium, potassium and calcium as the only metallic cation, they decrease in volume in all the solutions but they decrease most rapidly in distilled water containing lactose, less rapidly in the solution containing sodium and lactose, still less rapidly in the solution containing potassium and lactose, and least rapidly in the solution containing calcium and lactose.

3. If they are transferred from modified Ringer solution (0.002 M, pH 6.8) to Ringer solution containing lactose (0.2 M) and left 30 minutes, they decrease greatly in volume. If they are then transferred respectively to distilled water and to phosphate buffer solutions, containing respectively sodium, potassium and calcium (0.002 M, pH 6.8), they increase in volume in all the solutions, but they increase most rapidly in distilled water, less rapidly in the solution containing sodium, still less rapidly in the solution containing potassium and least rapidly in the solution containing calcium.

4. These results show that under some conditions monovalent as well as divalent cations cause decrease in the rate at which water passed into and out of *Amoeba proteus*. This is probably largely due to their action on permeability to water.

LITERATURE CITED

JACOBS, M. H., 1930. The influence of electrolytes on certain types of hemolysis. *Am. Jour. Med. Sci.*, **179**: 302–303.

MAST, S. O., 1928. Factors involved in changes in form in Amoeba. *Jour. Exper. Zoöl.*, **51**: 97–120.

MAST, S. O., AND W. L. DOYLE, 1934. Ingestion of fluid by Amoeba. *Protoplasma*, **20**: 555–560.

MAST, S. O., AND COLEEN FOWLER, 1935. Permeability of Amoeba proteus to water. *Jour. Cell. and Comp. Physiol.*, **6**: 151–167.

MAST, S. O., AND W. F. HAHNERT, 1935. Feeding, digestion, and starvation in Amoeba proteus (Leidy). *Physiol. Zoöl.*, **8**: 255–272.

MCCUTCHEON, M., AND B. LUCKÉ, 1928. The effect of certain electrolytes and non-electrolytes on permeability of living cells to water. *Jour. Gen. Physiol.*, **12**: 129–138.

THE CONTRACTILE VACUOLE IN AMOEBA PROTEUS (LEIDY)

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Metcalf (1910) maintains that there are scattered through the cytoplasm of *Amoeba proteus* numerous small round granules called microsomes or cytomicrosomes, and that the "contractile vacuole is surrounded by a layer of granules of the same size and appearance as the microsomes of the general cytoplasm." He says (p. 302): "When the vacuole is of moderate size these granules form a continuous layer, one granule thick," when it is large there are open spaces between the granules, and when it is small the layer is several granules thick. He maintains that during contraction these granules form a clump in which the new vacuole develops and consequently concludes that they "are connected functionally with excretion," and he therefore calls them "excretion granules." He admits, however, that the contractile vacuole can arise and function without the granules. He says (p. 307); "The results of operations upon Amoeba show that a new vacuole in an Amoeba fragment appears first and that only gradually the granules collect about it. The granules are not essential to the functional vacuole."

Mast (1926) confirmed Metcalf's contentions concerning the form, size and distribution of the granules in question, but he holds that the conclusion that they function in excretion is not well founded and that their aggregation on the surface of the contractile vacuole is a purely physical phenomenon. He designated them beta granules to differentiate them from somewhat similar but much smaller granules which he designated alpha granules.

Mast and Doyle (1935*a*, *b*) demonstrated that the beta granules have staining properties like the granules in other cells, known as mitochondria. They centrifuged amoebae and found that all the beta granules moved through the cytoplasm from the axis of rotation and that those on the contractile vacuole moved to the centrifugal surface and formed a considerable mass there, and they obtained some evidence indicating that the beta granules on the surface of the contractile vacuole are imbedded in a layer of substance which is somewhat more

viscous than the adjoining substance in the cytoplasm and moves with them during the process of centrifuging. They conclude that the beta granules function in transferring substances from place to place in the cytoplasm and that they probably thus facilitate excretion of substance by the contractile vacuole.

Methods

I made at various times numerous detailed observations on the contractile vacuole in *Amoeba proteus* under the best optical system obtainable (oil immersion apochromatic objective, 60 × and 90 ×, and compensating oculars, 10 ×, 15 × and 20 ×). All the observations were made on living specimens. Some of the specimens had been without food for several days and contained no food vacuoles and but little fat and relatively few granules and crystals. Some of these were greatly flattened by gradually removing water from under the coverglass. Under these conditions the contractile vacuole and the granules and other substances around it could be very clearly seen. The following results were obtained.

Results

The contractile vacuole is usually surrounded by a layer of beta granules (Metcalf's excretory granules). These granules are imbedded in a layer of substance which is optically distinctly different from the adjoining cytoplasm. It has a slightly yellowish tint and appears to consist of coagulated substance. It is more viscous and heavier than the rest of the cytoplasm for it moves to the centrifugal surface of the contractile vacuole when amoebae are centrifuged (Mast and Doyle, 1935*b*, Fig. 1). When the contractile vacuole is fully developed the granules around it usually form a layer one granule thick, with some spaces between the granules here and there. As the vacuole contracts these spaces disappear, the layer of granules becomes continuous and the layer of substance in which they are imbedded thickens. As it contracts further this layer becomes still thicker and the granules pile up on each other until there is, when contraction is complete, a distinct mass of substance of considerable size, in which a remnant of the vacuole can usually still be seen. That is, the vacuole usually does not completely disappear during systole and soon begins to enlarge again. If it disappears completely a new vacuole usually appears and develops in the mass of substance in which the old one disappeared (Fig. 1). This, with the exception of the layer of substance in which the granules are imbedded, is in full accord with Metcalf's views (1910).

At the surface of the contractile vacuole under the layer of substance containing the beta granules, there is a layer or membrane about

0.5μ thick which is optically well differentiated from the adjoining substance on either surface, for under favorable conditions a line indicating an interface can be clearly seen at both of these surfaces. The beta granules are never in close contact with this membrane.

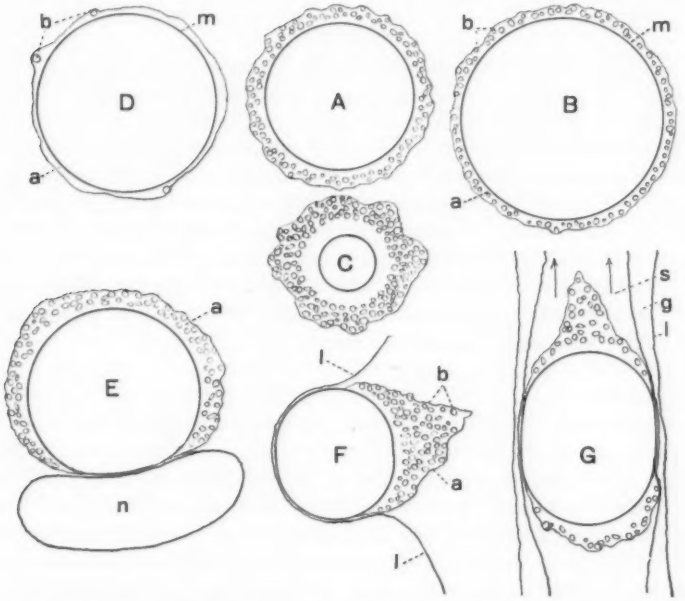

FIG. 1. Camera drawings of the contractile vacuole in *Amoeba proteus* showing the relation between the vacuole and the surrounding cytoplasm, under different conditions and in different phases. *m*, membrane on the surface of the vacuole; *a*, layer of differentiated cytoplasm around the vacuole; *b*, beta granules (mitochondria) imbedded in this layer; *l*, plasmalemma; *g*, plasmagel; *s*, plasmasol; *n*, nucleus.

A, contractile vacuole partially expanded; *B*, same vacuole fully expanded; *C*, same vacuole shortly after contraction; *D*, a vacuole which had only a few beta granules in the differentiated layer at the surface; *E*, *F*, *G*, contractile vacuoles which are in contact with other structures.

Note that the beta granules are not in contact with the membrane at the surface of the vacuoles, that they differ greatly in number in different vacuoles, and that the layer of differentiated cytoplasm in which they are imbedded becomes much thicker as the vacuole contracts. Note also that when the contractile vacuole comes in contact with other structures the differentiated layer around the vacuole is pushed aside.

They are always separated from it by substance in the layer in which they are imbedded. This separation is least when the contractile vacuole is fully developed and increases as it contracts (Fig. 1).

If the contractile vacuole comes in contact with an object, the layer

in which the beta granules are imbedded is pushed aside and the membrane around it comes in direct contact with the rest of the cytoplasm (Fig. 1). This shows that this layer is only loosely connected with the membrane.

The number of beta granules varies greatly and probably also the amount of substance in which they are imbedded. I have seen a considerable number of fully developed vacuoles which functioned normally in all respects, with only 10 or 12 granules at the surface (Fig. 1), and one in which there were no granules and no differentiated layer of substance. This indicates that these granules and the layer of substance in which they are imbedded have but little if any functional relation with the contractile vacuole. The layer of substance is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm.

Discussion

There is considerable confusion in reference to the ideas held by different investigators concerning the designation of the structure and the function of the granules and crystals in the cytoplasm of the protozoa.

Bütschli (1881, Abt. 1, S. 103) maintains that there are in freshwater rhizopods and other protozoa, numerous highly refractive bodies ("Körner") which vary greatly in size "von ausserster Kleinheit bis zu ziemlich ansehnlichen Dimensionen," and frequently appear in the form of rhomboid crystals, and that they are not composed of fat. He holds that these bodies are end-products of metabolism and he calls them "Excretkörnchen." He obviously, therefore, includes under this term various kinds of granules and crystals, that is, all which are highly refractive except those which are composed of fat.

Schewiakoff (1893) found in *Paramecium* small bodies he called "Exkretkörner." He studied these bodies in considerable detail and concluded that they consist of calcium phosphate and are optically active, "doppeltbrechend im polarisirten Lichte." He says (p. 32) "In Folge der Doppeltbrechung, welche bereits von Maupas ermittelt wurde, sind sie von anderen Plasmaeinschlüssen leicht zu unterscheiden." Schewiakoff's Exkretkörner therefore include only a portion of Bütschli's Excretkörnchen, in fact only a very small portion, for only a very small percentage of the granules and crystals in *Amoeba proteus* are optically active.

Metcalf (1910), as previously stated, maintains that there are on the surface of the contractile vacuole and scattered through the cytoplasm of *Amoeba proteus* small round granules. He calls them "microsomes," "Cytomicrosomes," or "excretory granules." Mast and

Doyle (1935) demonstrated that these granules are not optically active. They are therefore not like the Exkretkörner of Schewiakoff.

Taylor (1923, p. 266) says that certain granules which appear throughout the cytoplasm of *Euplotes* are apparently comparable with Metcalf's "cytomicrosomes" in *Amoeba* and *Opaline* (1910), Bütschli's "Exkretkörner" (1881), Maupas' "corpuscle refringents" (1883), and Schewiakoff's "Exkretkörner" in *Paramecium*.

Since Metcalf's cytomicrosomes differ radically from Schewiakoff's Exkretkörner, it is difficult to see how the granules in *Euplotes* can be comparable with both. Taylor does not say whether or not they are optically active but he calls them "crystaloidal, endoplasmic granules" and he says "I have never observed that they tend to aggregate about the contractile vacuole." They are therefore probably more nearly like Schewiakoff's Exkretkörner than Metcalf's "cytomicrosomes" or "excretory granules."

Taylor (p. 278) maintains that Khainsky (1911) observed granules pass through the walls of the food vacuoles into the cytoplasm and he says: "These crystalloids he [Khainsky] identifies with the Exkretkörner described at length by Schewiakoff. Khainsky thinks that eventually the granules are dissolved in the endoplasm and the solution is discharged to the outside by the contractile vacuole." I find that Khainsky presents evidence which indicates that granules pass from the food vacuoles directly into the surrounding cytoplasm, but I find nothing which indicates that he identified these granules with Schewiakoff's Exkretkörner or that he held that they are dissolved in the endoplasm and discharged through the contractile vacuole.

The fact that Bütschli designated the granules and crystals he observed "Excretkörnchen" clearly indicates that he held that they are involved in excretion. He says nothing, however, concerning this.

Schewiakoff maintains that the bodies he designated "Exkretkörner" dissolve in the cytoplasm and are excreted in fluid form by the contractile vacuole. He says (p. 55): "Es liegt demnach die Vermuthung nahe, dass die Exkretkörner im Protoplasma aufgelöst und im füssigen Zustande durch die kontraktile Vacuole nach ausen entleert werden."

Metcalf (1910) thinks that his "excretory granules" are "functionally connected with excretion." He gives nothing concerning the method involved, but his description of the relation between these granules and the action of the contractile vacuole shows clearly that he holds that they are not dissolved and discharged by the contractile vacuole. His conception concerning the method of function of these

granules consequently differs radically from Schewiakoff's concerning the method of function of his Exkretkörner.

Taylor (1923, p. 277) says: "Vacuoles (contractile) in Euplotes make their first appearance either from the coalescence of other extremely minute vacuoles that easily escape detection, or from the transformation of small vacuoles which contain one or more granules ('Exkretkörner'), or they arise de novo." I can see no essential difference between the first possibility and the third.

Concerning the second possibilities, Taylor thinks the granules in question may pass from the food vacuoles into the cytoplasm in accord with Khainsky's observations referred to above, that vacuoles may thus form around these granules, that the granules may then dissolve, and that the fluid vacuoles thus formed may develop into contractile vacuoles; and he holds that if this obtains the origin of the contractile vacuole is traceable to the food vacuole. The fact, however, that the contractile vacuoles continue to operate normally for days in the total absence of food, that long after all vacuoles containing food and the granules which have passed from them into the cytoplasm have disappeared, practically proves that they do not originate in this way.

Concerning the third possibility Taylor says: "Now it is conceivable that here and there in this organism 'fluid centers' normally arise which contain in solution substances (e.g. catabolic products) of a kind and concentration sufficient to induce the gelation of the surrounding plasm, thereby forming de novo normal vacuoles."

This possibility is in full accord with Metcalf's views concerning the origin of the contractile vacuole, except that he holds that the beta granules ("excretory granules") are involved. The fact, however, that under some conditions the contractile vacuole forms in regions where there are but few if any of these granules and that it develops normally at times when there are practically none on the surface of it, demonstrates that if they are at all functionally connected with the origin or the development of this vacuole, it is only in a very minor way.

It may be said then that while it is highly probable that the contractile vacuole originates in minute localized aggregations of fluid in the cytoplasm, nothing is known concerning the factors involved.

There is no evidence concerning the function of the relatively viscous layer in which the beta granules are imbedded on the surface of the contractile vacuole.

Summary

1. The contractile vacuole in *Amoeba proteus* contains at the surface a well differentiated membrane about 0.5μ thick. Adjoining this

membrane on the outside there is usually a layer of substance in which numerous beta granules (Metcalf's "excretory granules") are imbedded. This layer is more viscous and heavier than the adjoining cytoplasm. It is usually about 3μ thick when the vacuole is maximum in size and it becomes thicker during contraction.

2. The beta granules around the contractile vacuole vary greatly in number and the layer of substance in which they are imbedded varies greatly in thickness, without any apparent variation in the function of the vacuole. These facts indicate that neither the granules nor the layer of substance is involved in the function of the contractile vacuole, at least not directly.

3. The differentiation of a layer of substance on the surface of the contractile vacuole is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm.

4. There is much confusion concerning the ideas held by different investigators in regard to the nomenclature, the structure and the function of the various granules and crystals in the cytoplasm of the protozoa.

5. Bütschli's "Excretkörnchen" consist of various different kinds of granules and crystals found in protozoa. Schewiakoff's "Exkretkörner" are optically active bodies, nearly all of which, if not all, are crystals. Metcalf's "excretory granules" are not optically active. They are approximately 1μ in diameter and have staining properties like the mitochondria in other cells. The "Excretkörnchen" of Bütschli, the "Exkretkörner" of Schewiakoff and the "excretory granules" of Metcalf therefore differ radically in structure.

6. Bütschli says the "Excretkörnchen" are end products of metabolism but he says nothing concerning their elimination. Schewiakoff contends that the "Exkretkörner" are formed in the food vacuoles, pass out into the cytoplasm, dissolve there and are eliminated by the contractile vacuole. Metcalf holds that the "excretory granules" (Mast's beta granules) are permanent structures which function in excretion of substance by the contractile vacuole. Mast and Doyle maintain that they function in transporting substance through the cytoplasm.

LITERATURE CITED

BÜTSCHLI, O., 1881. Exkretkörner. Bronn's Die Klassen und Ordnungen des Tierreichs. Protozoa, Bd. 1, S. 103.

KHAINSKY, A., 1911. Zur Morphologie und Physiologie einiger Infusorien (Paramaecium caudatum) auf Grund einer neuen histologischen Methode. *Arch. f. Protist.*, **21**: 1–60.

MAST, S. O., 1926. Structure, movement, locomotion and stimulation in Amoeba. *Jour. Morph.*, **41**: 347–425.

MAST, S. O., AND W. L. DOYLE, 1935*a*. Structure, origin and function of cytoplasmic constituents in Amoeba proteus. I. Structure. *Arch. f. Protist.*, **86**: 155–180.

MAST, S. O., AND W. L. DOYLE, 1935*b*. Structure, origin and function of cytoplasmic constituents in Amoeba proteus with special reference to mitochondria and Golgi substance. II. Origin and function based on experimental evidence; effect of centrifuging on Amoeba proteus. *Arch. f. Protist.*, **86**: 278–300.

MAUPAS, E., 1883. Contribution a l'étude morphologique et anatomique des infusoires ciliés. *Arch. de Zool. expér. et gén.*, Ser. 2, **1**: 427–664.

METCALF, M. M., 1910. Studies upon Amoeba. *Jour. Exper. Zoöl.*, **9**: 301–331.

SCHEWIAKOFF, W., 1893. Über die Natur der sogenannten Exkretkörner der Infusorien. *Zeitschr. f. wiss. Zoöl.*, **57**: 32–56.

TAYLOR, C. V., 1923. The contractile vacuole in Euplotes; an example of the sol-gel reversibility of cytoplasm. *Jour. Exper. Zoöl.*, **37**: 259–290.

DURATION OF LIFE WITHOUT FOOD IN DROSOPHILA PSEUDOÖBSCURA

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Race *A* and Race *B* of *Drosophila pseudoöbscura* have been distinguished because they produce sterile male hydrids when crossed. Morphologically the races appear identical, and there are but few physiological characteristics that differentiate them. Thus, Poulson (1934) found that Race *A* flies have a shorter period of development than Race *B* flies. Dobzhansky [1] (1935) has studied the fecundity of both races at different temperatures and showed that during the lifetime Race *A* deposits more eggs than Race *B* at higher temperatures (25° C.), while at lower temperatures (19°, 14°) the relations are reversed. At all temperatures used Race *B* begins to oviposit later than Race *A*. In these experiments Dobzhansky observed also some indications suggesting that the longevity of Race *A* is greater than that of Race *B*. The present study is an attempt to secure further information bearing on this fact. In my experiments the measurements of the duration of life have been made in the absence of food. The data are therefore not necessarily comparable with those of Dobzhansky.

METHOD

Pearl and Parker (1924) state that any study involving longevity must be so conducted as to eliminate any desiccation; otherwise, the results are due to desiccation and not to the inherent factors which determine the length of life. Furthermore, they state that any technique which permits a fly to obtain water or any liquid even in small drops becomes a food study and not a starvation study. In the present work, however, the interest centered in the reaction of the two races to varying humidities and temperatures and not purely in the length of life at one optimum condition. Undoubtedly at a humidity of 95 per cent the flies took moisture condensing on the side of the tube.

Five bottles each of Texas (A) and Seattle—6 (B) cultures were

[1] The writer wished to express his indebtedness and sincere appreciation to Dr. Dobzhansky.

started on the twenty-ninth of May, each bottle containing 3 females and 5 males from stock that had been under laboratory conditions for some time. These were placed in the 19° (C.) cold room and transferred every 48 hours to fresh bottles, until four successive transfers had been made. Any cultures which showed a total number of pupæ under 100 or above 400 were discarded. On the fourteenth day of the culture's life, the paper was removed from the bottle and the pupæ were scraped from it, using a pair of small forceps. Any pupæ which were coated with food were discarded. The removed pupæ were placed in one-ounce vials, plugged with cotton and placed again at 19° to await the first hatching.

Poulson (1934) found that the maximum hatching occurred between the hours of 6 and 10 A.M., excepting Race *B* females which emerged in greatest numbers between 2 and 6 A.M. Since a fair percentage of the latter also hatch between 6 and 10 A.M. these four

TABLE I

Duration of life (in hours) at densities of 5 and 30 flies per one-ounce vial. Temperature 19° C., humidity 8 per cent.

Race	Five per vial		Thirty per vial		Difference
	$M \pm m$	n	$M \pm m$	n	
Seattle—6 ♀ (B)	71.70±1.18	90	69.60±1.10	60	+2.10±1.61
Seattle—6 ♂ (B)	74.90±2.01	70	68.80±1.44	60	+6.10±2.45
Texas ♀ (A)	102.57±1.20	105	97.05±1.17	150	+5.52±1.67
Texas ♂ (A)	104.88±1.76	75	98.67±1.33	90	+6.21±2.19

hours were chosen as the most convenient within which one might obtain representative populations. Each day of the hatch, therefore, all vials were emptied of imagines at 6 A.M., and at 10 A.M. the flies which had emerged were etherized and placed in sterilized glass vials, 20 per vial, sexes segregated. Each vial was covered with a piece of medium muslin, held tight by a rubber band. The flies were allowed to recover from the effects of etherization before being placed in desiccators. Using Obermiller's (1924) table of vapor pressures over salt solutions, the following chemicals were used: concentrated H_2SO_4 for 0 per cent, KOH (fused) for 8 per cent, $K_2CO_3 \cdot 2H_2O$ for 43 per cent, and K_2SO_4 for 95 per cent humidity.

After recovery the vials were numbered and placed upright in the desiccator, using a piece of white cardboard to hold the single row of vials close to the glass of the desiccator so that counts could be taken without removing the desiccator top.

Results

An attempt was made to determine the length of life of *D. pseudoöbscura* at two population densities. Pearl and Parker (1924) found that, on food, densities from 35 to 45 flies per bottle were optimum for *D. melanogaster*. At lower and especially at higher densities a reduction of the life span was observed. The data for *D. pseudoöbscura* are presented in Table I.

Table II

Duration of life at two temperatures and four different humidities

		Humidity 0 Per cent		Humidity 8 Per cent		Humidity 43 Per cent		Humidity 95 Per cent	
	Race	$M \pm m$	N	$M \pm m$	N	$M \pm m$	N	$M \pm m$	N
Temperature, 19°	Seattle—6 ♀	70.38 ±1.91	60	77.64 ±1.62	120	94.28 ±1.01	238	101.60 ±1.90	51
	Sequoia—8 ♀	63.10 ± .755	60	65.12 ±1.77	161	81.00 ±1.28	180	104.25 ±1.39	48
	Texas ♀	85.76 ±1.10	60	97.20 ± .77	55	102.97 ± .69	396	127.60 ± .98	53
	Mara—3 ♀	67.45 ±1.14	129	85.80 ±1.93	130	100.68 ±1.47	139	110.52 ±2.02	60
	Seattle—6 ♂	74.0 ±1.39	60	74.40 ±1.14	131	99.10 ±2.415	210	92.40 ± .13	70
	Sequoia—8 ♂	66.75 ± .955	60	66.37 ±1.15	159	89.22 ±1.20	160	102.00 ± .86	40
	Texas ♂	74.46 ±1.87	60	91.32 ±1.06	100	104.93 ± .80	254	111.0 ±1.33	40
	Mara—3 ♂	73.57 ±1.02	110	87.00 ±1.88	97	99.84 ±2.10	120	113.10 ±1.90	40
Temperature, 24°	Seattle—6 ♀			53.52 ± .90	120	55.16 ±1.80	100	60.50 ±1.10	40
	Sequoia—8 ♀	35.46 ± .45	100	45.60 ± .733	80	57.08 ±1.37	80	65.10 ±.705	100
	Texas ♀	57.30 ± .945	80	63.50 ±1.06	100	73.84 ±1.45	60	85.80 ±1.67	40
	Mara—3 ♀	35.58 ± .498	100	50.88 ± .657	220	63.60 ±1.63	80	74.40 ±2.36	80
	Seattle—6 ♂			50.79 ± .98	120	54.68 ± .566	120	62.76 ± .115	40
	Sequoia—8 ♂	37.80 ± .54	60	43.84 ± .857	80	56.05 ±1.25	60	71.66 ± .945	80
	Texas ♂	59.52 ±1.36	60	62.57 ± .917	100	75.46 ±1.43	80	87.90 ±1.43	80
	Mara—3 ♂	38.40 ± .39	80	52.80 ± .622	200	71.55 ±1.36	60	76.83 ±1.65	60

The data presented in Table I tend to show that the lower density is more favorable for *D. pseudoöbscura* than the higher one. They show furthermore that the duration of life of Race *A* is greater than that of Race *B*. The latter conclusion must, however, be checked in several respects. First of all, the fact that one strain of Race *A* differs from one strain of Race *B* does not necessarily indicate that the difference is characteristically racial; it may be a property of the particular strains used. Moreover, a difference observed under one set of conditions may be obliterated or even reversed under other conditions. The longevity of the flies has therefore been studied at temperatures 19° and 24° C., and at humidities of 0 per cent, 8 per cent, 43 per cent and 95 per cent (Table II). Finally, a variety of strains of both races coming from different parts of their distribution area has been studied (Table III).

Among the strains used, Texas, Mara, Julian, Oaxaca, Olympic, Pavilion, and Taos belong to Race *A*, and Seattle, Sequoia, Campbell, Crater, Quilcene, and Quesnel to Race *B*. It can be seen from Tables

II and III that in general Race *A* tends to be more long-lived than Race *B*. Nevertheless, some intra-racial variation is observed. Thus, the longevity of the Texas strain is greater than that of the Mara strain; the Quesnel strain is superior to other Race *B* and to some Race *A* strains. It may be noted that at 95 per cent and 43 per cent humidity Race *A* strains are in general superior in longevity to Race *B*. At lower humidities, especially at 0 per cent, a greater degree of overlapping between races is observed. Qualitatively, the

TABLE III

Duration of life in nine strains of *D. pseudoöbscura* at 24° C. and three different humidities

		Humidity 0 Per cent		Humidity 43 Per cent		Humidity 95 Per cent	
	Race	$M \pm m$	N	$M \pm m$	N	$M \pm m$	N
Race *B*	Campbell—4 ♀	40.13± .770	90	48.85±.615	80	68.57±1.40	80
	Crater—2 ♀	38.09± .628	60	46.80±.514	60	61.86±1.05	60
	Quilcene—4 ♀	40.00± .740	90	47.04±.648	60	66.24±1.68	40
	Quesnell—5 ♀	46.91± .655	60	56.12±.727	68	69.43±1.585	60
	Campbell—4 ♀	41.02± .634	103	51.64±.605	80	66.62±1.54	60
	Crater—2 ♂	38.10± .715	60	50.00±.595	60	62.15±1.095	82
	Quilcene—4 ♂	38.84± .500	120	43.08±.468	60	57.30±1.49	60
	Quesnell—5 ♂	47.07± .475	60	55.41±.495	80	59.10±1.295	60
Race *A*	Julian E—6 ♀	53.02± .770	80	63.72±.755	60	83.62±1.96	60
	Oaxaca—4 ♀	43.08± .707	85	50.64±.741	60	90.54±1.86	80
	Olympic—2 ♀	46.62± .684	80	61.58±.787	40	92.05±1.69	80
	Pavillion—5 ♀	49.25± .705	89	66.37±.455	100	88.88±1.54	60
	Taos—1 ♀	49.89±1.18	70	56.40±.800	60	70.10±1.54	80
	Julian E—6 ♂	47.02± .655	100	60.00±.482	80	76.44±2.21	60
	Oaxaca—4 ♂	45.32± .644	100	56.57±.616	60	90.73±2.41	60
	Olympic—2 ♂	46.85± .632	90	65.43±.880	40	92.93±1.68	80
	Pavillion—5 ♂	50.00± .806	60	64.87±.545	80	89.73±2.01	40
	Taos—1 ♂	51.78± .955	60	61.60±.641	80	64.56±2.00	60

effects of humidity and temperature are identical for all strains studied. The duration of life is greater at the lower temperature (19°) than at the higher one (24°), and at higher humidities than at the lower ones.

The two races of *D. pseudoöbscura* differ in their geographical distribution. Race *B* is restricted to the northern part of the Pacific Coast, while Race *A* lives much further eastward and southward than Race *B* (Dobzhansky, 1935, 1937). As far as our data show, the effects of humidity and temperature on the duration of life in the absence of food have little bearing on the geographical distribution of

the two races. Further studies, especially those on the duration of life in the presence of food, may conceivably throw more light on this problem.

Summary

1. In the absence of food, the duration of life of *Drosophila pseudoobscura* is greater at lower temperatures, greater humidities, and lower population densities studied.

2. With temperature, humidity, and population density being kept constant, Race *A* lives longer than Race *B*. These differences between the races is more pronounced at higher than at lower humidities.

Literature Cited

Dobzhansky, Th., and R. D. Boche, 1933. Intersterile races of Drosophila pseudoöbscura Frol. *Biol. Zentrlbl.*, **53**: 315.

Dobzhansky, Th., 1935. Fecundity in Drosophila pseudoöbscura at different temperatures. *Jour. Exper. Zoöl.*, **71**: 449.

Dobzhansky, Th., 1937. Genetic nature of species differences. *Am. Nat.*, **71**: 404.

Obermiller, J., 1924. Equilibrium humidities over saturated salt solutions. *Zeitschr. f. Physik. Chem.*, **109**: 145.

Pearl, R., and S. L. Parker, 1924. Experimental studies on the duration of life. X. The duration of life of Drosophila melanogaster in the complete absence of food. *Am. Nat.*, **58**: 193.

Poulson, D. F., 1934. Times of development of the two races of Drosophila pseudoöbscura. *Jour. Exper. Zoöl.*, **68**: 237.

THE LIFE HISTORY OF A TREMATODE (LEVINSENIELLA CRUZI?) FROM THE SHORE BIRDS (LIMOSA FEDOA AND CATOPTROPHORUS SEMI-PALMATUS INORNATUS) [1]

(Contributions from the Scripps Institution of Oceanography New Series, No. 19).

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INTRODUCTION

Lebour (1911) has described a cercaria (*C. ubiquita*) from British snails (*Paludestrina stagnalis*, *Littorina obtusata* and *L. rudis*). She found "Cercariæ exactly corresponding with *C. ubiquita*, but without the glands, inside the tissues of *Carcinas mœnas*" and similar metacercariæ were found by her in *Cancer pagurus*, the third host in the cycle being presumably the herring gull (*Larus argentatus*). The same or a closely related larva has been found by Stunkard (1932) in *Littorina rudis* and *L. littorea* at Roscoff, France, and Rees (1936) has observed similar larvæ in these snails and in *L. obtusata* at Aberystwith, England. The adult form of these larvæ is supposed to be some species of *Levinseniella*, but thus far the various steps in the life history have not been definitely established.

In a study of the trematodes of the La Jolla region on which I am engaged I have found a stylet cercaria infesting the snail *Olivella biplicata* which differs in some particulars from those of Lebour and Stunkard, and whose probable adult form I have located in the shore birds (*Limosa fedoa* and *Catoptrophorus semipalmatus inornatus*), while the metacercaria parasitizes the sand crab (*Emerita analoga*).

I will first describe the cercaria, the metacercaria and the adult worm, and then give the evidence on which my conclusions regarding the life history are based.

THE CERCARIA

The cercaria (Fig. 1) is one of the spineless xiphidio-cercariæ, possessing a stylet from 24 to 32 μ long, and a set of eight unicellular penetration (?) glands, but no pigment spots. In fixed specimens the body averages 165 $\times$ 46 μ and the tail 124 μ in length. It has an oral

[1] The following study has been conducted at the Scripps Institution of Oceanography, to whose directors, Dr. T. Wayland Vaughan and Dr. H. U. Sverdrup, and other members of the staff I am indebted for many courtesies.

sucker 34 μ in average diameter, but no acetabulum, or gut. The excretory bladder is bifurcate, but further details of the excretory system are not evident.

Perhaps the most interesting feature of the larva are the penetration (?) glands, which are in two sets, an anterior set of four larger, and a posterior one of four smaller glands. Their arrangement is shown in Fig. 1, where they are designated by numbers 1–8 respectively. Each gland has a duct which follows a more or less sinuous course depending on the state of contraction of the worm, and which opens at the anterior border of the sucker. Lebour states that there

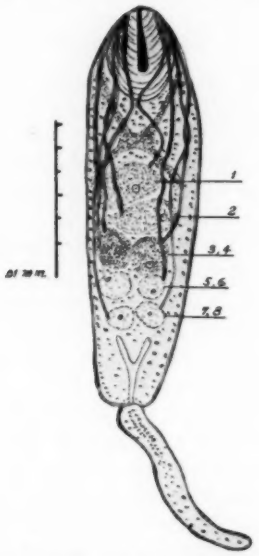

FIG. 1. Cercaria showing stylet, sucker, nerves, excretory bladder and glands (1–8). For description of latter, see text. Outline with camera, details free-hand.

are but four ducts for the group of glands in her larva, but their close apposition and sinuous course renders their individual identification difficult and might easily lead to confusion about their actual number. My specimens, when stained with neutral red, show clearly one duct for each gland. Both glands and ducts stain intensely in this stain.

These glands differ markedly; not only in size, but also in the physical and apparently chemical character of their contents. Glands No. 1 and 2 are finely granular and do not ordinarily take an eosin stain. Occasionally Nos. 1 and 2 stain lightly in eosin but much less

intensely than Nos. 3 and 4. Numbers 3 and 4 are also finely granular, though the granules are perhaps a trifle coarser than in Nos. 1 and 2, but they take the eosin stain intensely. Glands 5–8 stain very lightly. They contain only a few eosinophile granules which are scattered through the meshes of a rather open reticulum, so that the whole cell appears as an open space in the sections with what is usually a pycnotic,

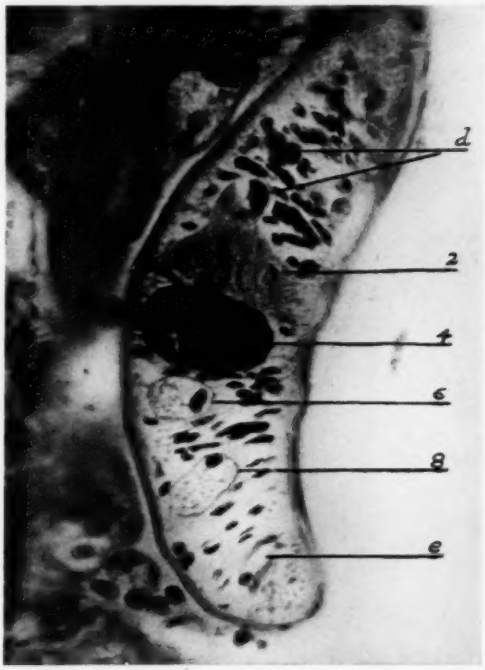

FIG. 2. Photomicrograph of long section of cercaria showing glands 2, 4, 6 and 8 (see Fig. 1 and text). *d*, ducts; *e*, excretory bladder; × 860.

heavily stained nucleus near the center. The nuclei in all eight glands, however, vary widely, sometimes being pycnotic and densely stained, and at other times vesicular and so lightly stained as to be scarcely visible. Especially is the latter true of glands 1 and 2. That these differences are real and not artificial is indicated by the fact that they may occur in neighboring larvæ in the same sporocyst. These structural and staining differences are evidence of functional differences in the two sets of cells 1–4 and 5–8 respectively. It is probable that each set elaborates a different secretion, for were the differences in structure

and staining capacity due to different stages in the elaboration of the *same* secretion the two sets of cells should vary in different individuals. But this is apparently not the case; each set is distinct in every cercaria. The difference in eosinophily between cells 1, 2, and 3, 4 respectively is probably explicable by differences in the stage of development of the same secretion, but this will not explain the difference between sets 1–4 and 5–8.

Stunkard (*loc. cit.*, p. 337) says of his larva that "the cells do not stain with neutral red, but the secretory granules are clearly visible . . . (which granules) stain a deep blood red in the terminal portion of the ducts and frequently accumulate there to form enlargements," while Rees (*loc. cit.*, p. 622) says that "only the two anterior pairs (of cells) and their ducts . . . (stain) a deep red. The remaining cells and their ducts have never been observed to take up the intravital stain." All eight cells in my larva stain uniformly in neutral red, the differences appearing in the material stained with eosin.

It is difficult to harmonize these various observations with one another. They *may* indicate specific differences in the cercariæ themselves but it is not impossible that they are due to functional differences in the glands at different times and under different conditions. Possibly they are the effect of different hosts.

The infestation of the snail is considerable, 6 out of 98 specimens examined being infested.

The shedding of the cercariæ appears to be a seasonal matter, although other, as yet obscure factors may affect this. In February and March, 1935, cercariæ were easily obtained by isolating snails from the beach near the Scripps Institution, whereas from November, 1936 to January, 1937 isolation of several hundred snails produced no cercariæ. In May and June of 1937, a few cercariæ were obtained from several hundred snails, but in July and August, 1937, isolation of several hundred snails failed to produce any. That some of the apparently sterile snails were infested, however, was proven by dissection of several specimens. Lebour (*loc. cit.*) also has observed that snails are free from *C. ubiquita* in winter, while their walled cysts (metacercariæ) are present in crabs only during spring and summer and early autumn. I have not made sufficient study of this matter, however, and hence cannot define definitely the seasonal cycle of the life history, if such exists, which appears to be the case.

The Metacercaria

The cercariæ in the crab lack tails, but whether these are lost before or after entrance I cannot say. The former appears more likely,

however, as the tails are frequently detached from the cercariæ while swimming. Soon after entrance the glands and ducts become broken down and are identifiable only as irregular masses which still stain in neutral red. Within two or three days the cercaria secretes a cyst within which it curls up and begins its development into the metacercaria.

This naturally varies in size with age, the older ones reaching a diameter of 0.3 mm. with a cyst wall about .025 mm. thick. It is very slightly ovoid in shape and is found in the connective tissue of the crab, chiefly among the liver tubules. Practically every crab examined, in the La Jolla region, over 6 mm. in length is infested. I have not determined the maximum number of cysts which one crab may carry, but it is probably several dozen.

The Adult

The adult worm agrees closely in all important details with the description of the genus *Levinseniella* as given by Jägerskiöld (1900). The outline is narrower in front and broader behind, and when much expanded the body may be slightly constricted just in front of the acetabulum. This latter is located about two-thirds the length of the body from the anterior end. The anterior two-thirds of the body is spinose, the spines gradually diminishing in size and disappearing at about the level of the acetabulum.

Judging from the figure (unlabeled) and the description (in Portuguese) of *L. cruzi* by Travassos (1920) my specimens belong to this species, although the difference in the orders of the hosts (*Anseres* and *Charadriformes*) and in locality (California and Rio de Janeiro, Brazil) render this uncertain. As shown in Table I, the dimensions of my specimens correspond rather well with those of *L. cruzi*,[2] the chief differences being in the size of the oral sucker, which in my specimens averages 0.061 mm. in diameter while in the former it averages from 0.092 to 0.113 mm., and in the length of the ceca (0.134 and 0.163 to 0.241, respectively).

Perhaps the most striking feature of agreement between the two is in the size of the eggs, which average larger in both than in any other species known to me. The average of 24 eggs from my specimens fixed in aceto carmine is 0.036 by 0.017 mm., while in *L. cruzi* they average from 0.021 to 0.035 by 0.012 to 0.014. When fairly extended in life my specimens range from 0.55 to 0.9 mm. in length, while *L. cruzi* ranges from 0.609 to 0.713 mm. While there thus appear to be certain minor differences between my worms and those of

[2] Presumably measured from fixed material although Travassos does not so state.

TABLE I

Table I showing average measurements in μ of fresh and preserved specimens, with the number of specimens averaged for each measurement in parentheses. With the exception of five eggs, all preserved specimens were fixed in aceto-carmine. There is some overlap in the two sets, i.e. a few specimens were measured first in the fresh, and then in the preserved condition. Column 3 shows the measurements of *L. cruzi* from Travassos (*loc. cit.*).

	Fresh	Preserved	*L. cruzi*
Length	692 (14)	676 (15)	609–713
Width	193 (14)	303 (15)	222–313
Oral sucker	61 (12)	61 (14)	92–113
Pre-pharynx	37 (3)	36 (5)	14–56
Pharynx	33×21 (8)	32×23 (9)	35×53–21×35
Post-pharynx	168 (4)	170 (10)	63–120
Ceca	112 (7)	134 (10)	163–241
Acetabulum	54 (15)	55 (15)	56–71
Acetab. distance (from ant. end)	485 (9)	457 (9)	
Penis diameter	23 (9)	23 (9)	
Seminal vesicle	67×39 (5)	67×38 (8)	
Ovary	74×49 (12)	71×54 (15)	56–92
Right testis	97×58 (12)	93×69 (14)	
Left testis	90×61 (12)	89×65 (14)	
Egg		36×17 (24)	35×14–21×12

Travassos, they are insufficient in my judgment for separating them specifically, and I am therefore, in spite of the difference in hosts and locality, provisionally assigning my specimens to his species.

The Life History

The evidence upon which my conclusions regarding the life history of this worm are based is briefly as follows:

(1) the presence in the sand crabs of stylet cercariæ, and of all stages in the developing metacercariæ from that of the cercaria surrounded by a very thin cyst, but still having the stylet and remnants of the penetration (?) glands to those of the trematode, which resembles the adult in every way save for the presence of eggs in the uterus;

(2) the experimental infestation of crabs with cercariæ;

(3) the presence in the birds of adult worms which resemble in every way the excysting metacercariæ, save for the presence of eggs in the uterus, and the presence of both encysted and excysted metacercariæ in the birds' stomachs after they have fed on sand crabs.

There is apparently in nature a universal infestation of all crabs more than 6 mm. long. I have not recorded the number of crabs which I have examined (50–100), all of which were infested. It is probable that infestation begins when the crabs are about 5 mm. long as I have found specimens of this size containing cercariæ. Until they reach a length of at least 5 mm. the crabs are less frequently infested. Of 30 specimens of this size or less which I examined in nature only 8 contained cercariæ. It is evidently a matter of age, and consequently length of time of exposure, which determines the infestation, for the larger specimens are more heavily infested than the smaller ones, and very young crabs are susceptible to experimental infestation. The season of the year is possibly also an important factor, for, as already shown,[3] the deposition of cercariæ by the snails may be dependent on the season.

I have performed three sets of experiments in an attempt to demonstrate the transfer of cercariæ from snail to crab, the results of which are summarized in Table II.

In the first of these a number of cercariæ were obtained from snails which shed them naturally, transferred to a finger bowl with ten crabs from 4 to 5 mm. long, and left for from three to four days, after which, examination of the latter showed that seven out of eight (all of which were examined at the time) contained from one to four cercariæ each while the remaining crab which was dissected 42 days later was negative.

[3] See page 322.

As a control 15 crabs collected on the beach within four weeks of this experiment gave a ratio of 5 infested to 10 non-infested specimens. An experimental infestation of seven out of nine young crabs, while 10 of 15 in nature were uninfested, has a probability, on the basis of chance alone, of .03333.[4]

A second group of experiments consisted in holding for one or two days 18 young *Emerita* in finger bowls, together with cercariæ which had just been shed by snails. Seven of these were subsequently found to be infested. In the control for this experiment, out of 20 young crabs none were infested. In this experiment and its control the crabs were kept for several days previously in running sea water which was filtered through several inches of sand, thereby eliminating the likelihood of an accidental infestation. The probability of this result occurring on the basis of chance alone is .0021.[4] In explanation of

TABLE II

Showing results of experimental infestation of *Emerita* with the cercaria of *Levinseniella cruzi* (?) [5]

Series 1		Series 2		Series 3	
Experiment *Emerita* exposed to cercariæ		Experiment *Emerita* exposed to cercariæ		Experiment *Emerita* exposed to *Olivella*	
Positive	Negative	Positive	Negative	Positive	Negative
7	2	7	11	17	18
Control *Emerita* collected on beach		Control *Emerita* in filtered sea water		Control *Emerita* in unfiltered sea water	
Positive	Negative	Positive	Negative	Positive	Negative
5	10	0	20	6	40

the low ratio of infestation (7 : 18) in this series of experiments, as compared with the high ratio (7 : 9) in the former series, it may be said that the number of cercaria per crab was much smaller in the latter than in the former series.

In a third series of experiments *Emerita* were held from one to several days in jars [6] containing infested snails for comparison with crabs held in running sea water. The result of this experiment was an infestation of 17 out of 35 crabs in the experimental jars compared with 6 out of 46 in the control. The probability of this result on a purely chance basis is .0003.[7]

[4] Computed from McEwen (1929).

[5] In determining the presence or absence of parasites in the crabs the factor of length of examination is essential. I adopted a minimum of 12–15 minutes for the smaller (5–6 mm.) specimens and of 15–20 for the larger (10–15 mm.) ones.

[6] Some of these were supplied with running sea water. In others the water was standing, but aerated.

[7] Computed from McEwen (1929).

In evaluating this series of experiments it should be observed that in many of them only a very meagre number of cercariae were furnished a considerable number of crabs in a volume of from two to eight liters of water, hence the chance of any individual crab receiving a cercaria was rather small. The infestation of a considerable number of crabs in the controls (6 in 46) is difficult to explain. Very rarely cercariae occurred in the laboratory water supply, but straining through cotton a small stream of water from a tap for about forty hours produced no cercariæ, so that their chance occurrence in the control aquaria can hardly be explained thus. In many cases the control aquaria were standing on the same table with jars which contained possibly infested snails and it is possible that cercariæ from the latter may have infested the controls. In one case in particular a control jar of *Emerita* collected on August 17, 1937 contained recently infested crabs until August 28, during which time a jar of snails was standing on the same table; but straining the water from this jar through fine bolting cloth on this date failed to produce any cercariæ. On August 30 and September 1 after removal of this jar an examination of ten crabs from this control revealed no recent infestations.

The source of the occasional infestation of the controls therefore remains uncertain, but in any case the difference in the infestation ratios between experiments and controls in this series is highly significant.

Taken collectively the experiments give a result of 31 infestations out of 62 tests, while the controls give 11 out of 81 tests; and these include 5 out of 15 exposed to natural infestation on the beach. This result, on the basis of chance alone, has a probability of .0000013.[8]

Larvæ similar to those in the sand crabs occur in the fiddler crabs (*Uca crenulata*) from Mission Bay, an inlet of the sea near La Jolla. Doubtless, many other crabs of the vicinity harbor the same or related larvæ, for Lebour (*loc. cit.*) and Stunkard (*loc. cit.*) in Europe have found related metacercariæ in several species of crabs.

The extent of infestation of the willet and godwit by this parasite is rather limited. Only toward the end of this study did I differentiate between this species and two other related ones so that I cannot give any figures on the percentage of infestation. However, the former is usually, if not always, greatly outnumbered by what appear to be two other species of *Levinseniella*. In at least one case, that of a marbled godwit, it was apparently absent, but time did not permit an examination of the entire gut of this bird so that possibly a few specimens escaped observation.

[8] Computed from McEwen (1929).

McMullen (1935) gives some data which are of interest in this connection. In experimental infestations of *Amia calva* with *Macroderoides typicus* he found the fish heavily infested a few days after feeding infested material, but after several months the infestation was only slight. Thus one fish examined after 22 days contained by actual count at least 1392 flukes, while another fish examined 202 days after infestation contained only 33. He believes that "This enormous loss . . . was probably . . . due to crowding" (*loc. cit.*, p. 375). And Sarles and Stoll (1935, p. 290) state that "cats . . . carrying natural infestations of *Toxacara cati* were found to possess a uniformly high degree of resistance against attempts to superimpose infection with this ascarid." What limits the degree of infestation in wild animals, many of whom, as is the case with these shore birds, have unlimited opportunity for acquiring it, is as yet an unsolved problem. It may be, as suggested by Stunkard (1930) and Stoll (1929), analogous to the development of immunity by animals to bacterial infection. In the case of *L. cruzi* and the shore birds it is possible that the usually abundant infestations with other species limits the number of the former. It does not, however, apparently interfere with extensive infestation by a *Maritrema sp.* which the birds probably receive from the sand flea (*Orchestoides*), which forms a considerable part of their diet.

In trying to follow the life history of this worm I was at first misled by finding excysted individuals in the surf perches (*Embiotoca, Abeona*) after the latter had eaten sand crabs. It was only after performing several experiments that I discovered that the infestation of the fish is only temporary. It is rather remarkable, however, that a parasite of a homoiothermal animal should excyst and exist, even for a brief period in a poikilothermal form. My observations on this point are not solitary, however, for Linton (1928) reports *Levinseniella adunca* from a sanderling (*Crocethia alba*) which "appeared to be identical with a species found in the toad fish (*Opsanus tau*)" (*loc. cit.*, p. 21).

The last link in the life history of this worm is not yet made. In spite of repeated fecal examinations of both wild and captive birds, I have not succeeded in finding either eggs or miracidia. The eggs in utero appear never to have developed beyond an early cleavage stage. I have one probable record of a miracidium in the snail, but as my observations were very brief, the organism being lost in transfer to a slide, I am not able to assert positively that it *was* a miracidium, much less to determine whence it came. There must, however, be

some period when the birds are shedding eggs or miracidia. This I hope subsequent study will reveal.

Summary

The life history of a trematode (*Levinseniella cruzi?*) from the shore birds (*Limosa* and *Catoptrophorus*) and the structure of its cercaria have been described in some detail. The probable life history is as follows:

The miracidium has not yet been certainly observed. The cercaria, which belongs to the ubiquita group of Lebour, inhabits the snail, *Olivella biplicata*. From here it passes to the sand crab, *Emerita analoga*, which latter, when eaten by the birds, infests them with the adult trematode. The degree of infestation of the snail is considerable, but the cercariæ are shed infrequently, possibly at definite seasons.

Practically all crabs over 6 to 7 mm. in length are infested, but for some as yet undetermined reason, the infestation of the birds is light.

BIBLIOGRAPHY

Jägerskiöld, L. A., 1900. Levinsenia (Distomum) pygmæa Levinsen, ein genitalnapftragendes Distomum. *Centralbl. f. Bakt.*, Abt. 1, **27**: 732.

Jägerskiöld, L. A., 1909. Kleine Beiträge zur Kenntniss der Vogeltrematoden. *Centralbl. f. Bakt.*, Abt. 1, **48**: 302.

Lebour, M. V., 1911. A review of the British marine cercariæ. *Parasitol.*, **4**: 416.

Linton, E., 1928. Notes on trematode parasites of birds. *Proc. U. S. Nat. Mus.*, **73**: 1.

McEwen, G. F., 1929. Methods of estimating the significance of differences in, or probabilities of fluctuations due to random sampling. *Bull. Scripps Inst. Oceanography*, Tech. Ser., **2**: 1.

McMullen, D. B., 1935. The life histories and classification of two allocreadiid-like plagiorchids from fish, Macroderoides typicus (Winfield) and Alloglossidium corti (Lamont). *Jour. Parasitol.*, **21**: 369.

Rees, W. J., 1936. Note on the ubiquitous cercaria from Littorina rudis, L. obtusata and L. littorea. *Jour. Mar. Biol. Ass'n.*, **20**: 621.

Sarles, M. P., and N. R. Stoll, 1935. On the resistance of the cat to superimposed infection with the ascarid, Toxocara cati. *Jour. Parasitol.*, **21**: 277.

Stoll, N. R., 1929. Studies with the strongyloid nematode, Hæmonchus contortus. Acquired resistance of hosts under natural reinfection conditions out-of-doors. *Am. Jour. Hygiene*, **10**: 384.

Stunkard, H. W., 1930. The life history of Cryptocotyle lingua (Creplin), with notes on the physiology of the metacercariæ. *Jour. Morph.*, **50**: 143.

Stunkard, H. W., 1932. Some larval trematodes from the coast in the region of Roscoff, Finistère. *Parasitol.*, **24**: 321.

Travassos, L., 1921. Contribuição para o conhecimento da fauna helmintologica brazileira IX, Sobre as especias da subfamilia Microfalinae Ward 1901. *Arch. Escol. Sup. Agr. Med. Vet. Nictheroy*, 4 (1920) 1921, pp. 85–91.

THE EFFECTS OF ULTRA-VIOLET RADIATION OF λ2537A UPON CLEAVAGE OF SEA URCHIN EGGS[1]

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The effect of ultra-violet radiation upon the division rate of various cells has been the subject of a number of investigations, but the results are not in complete agreement, some investigators claiming acceleration (e.g., Coblentz and Fulton, 1924; Hinrichs, 1928; Alpatov and Nastjukova, 1933), others retardation (*e.g.*, Hertel, 1905; Gates, 1929; Oster, 1934; Chase, 1937), still others acceleration or retardation depending upon the wave-length and dosage (*e.g.*, Bovie and Hughes, 1918; Hughes and Bovie, 1918; Hutchinson and Ashton, 1929).

The disagreement may be more apparent than real, the results depending upon the wave-length of the ultra-violet, the dosage and the organism used. Quantitative data are needed to throw further light upon the problem. The following paper is an attempt to gather such data on the effects of one wave-length, 2537A, upon cleavage of sea urchin eggs, this material being chosen because the self-contained food supply greatly simplifies control of the environment. Work is planned at each of the other wave-lengths of ultra-violet light represented in the spectrum of the quartz mercury arc.

Materials and Methods

The sea urchins (*Strongylocentrotus purpuratus* Stimpson) were collected during the winter breeding seasons of 1935 and 1936 at Moss Beach and Pacific Grove, California. Eggs were obtained after natural spawning or by excision of the ovaries and from 25 to 100 eggs were placed in each 1″ watchglass containing 3/4 cc. sea water. The sperm suspension was determined each time by tests, successive dilutions being made until 100 per cent fertilization was achieved without overinsemination, and since almost all eggs cleaved normally, polyspermy was probably rare. Only eggs in which practically 100 per cent showed fertilization membranes within two minutes after insemination were used in this research.

In the case of eggs kept at 14–16° C., the first cleavage occurred in about two hours following insemination, the second after another

[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

hour, and the successive cleavages to the 64-celled stage at about hourly intervals. Within 20 hours after insemination the actively swimming blastulæ had been formed and in about another 24 hours gastrulation was complete, having begun some 12 hours earlier. Plutei formed within 4 days after insemination although the arms did not begin to elongate for another 24 hours. Since controls showed normal development to the pluteus, the conditions were considered satisfactory for the investigation.

For the first series of studies a mercury-argon discharge tube which emits about 88 per cent of the total output in the visible and ultraviolet at λ2537A (Coblentz, 1931; Leighton and Leighton, 1935) was employed since its high intensity enables one to give an effective dose of radiation in a short time and renders feasible certain experiments otherwise impossible. The intensity of the radiations, after screening out the infra red rays by a suitable water filter, was shown by thermopile measurements to be relatively constant for the period of investigations.

For experiments where pure light of λ2537A was needed the radiations from a water-cooled quartz mercury arc were passed through a natural quartz monochromator and the light of the desired wavelength was focused on the quartz cell containing the eggs. The apparatus used was in general similar to that previously described (Giese and Leighton, 1935).

The line thermopile (type described by Leighton and Leighton, 1932, p. 1884) used in series with a D'Arsonval H.S. galvanometer, was calibrated against Bureau of Standards Lamps C-211 and C-212. The thermopile factor for λ2537A was calculated to be 24.10 ergs/sec./cm. galvanometric deflection.

Experimental

Irradiation of Eggs Just Before the First Cleavage

In the first series of experiments the eggs which had been inseminated 90 minutes previously were irradiated for 1, 2, 4, 8, 16, 32, and 64 seconds at a distance of 31 mm. from the center of the mercury argon tube, and for 1, 4 and 16 seconds at a distance of 248 mm. from the center of the tube (to give exposures approximately equivalent to 1/64, 1/16, 1/4 second). In one series exposures approximately equivalent to 1/8 and 1/32 second were also given. Examinations were made at intervals of a half hour or an hour, depending upon circumstances, and the stages in development recorded. A typical set of data from a series of 3 experiments is plotted in Fig. 1.

It is clear from Fig. 1 that delay in cleavage is proportional to dosage and that even 1 second irradiation is sufficient to delay development for a long time, 1/4 second for a slight period of time. However, the protoplasm of the egg hastily repairs injury from lesser dosages, for example, the rate of cleavage of eggs irradiated 1/8, 1/16, 1/32 and 1/64 second was at no time lower than that of controls. But in no case was an increased rate of cleavage observed.

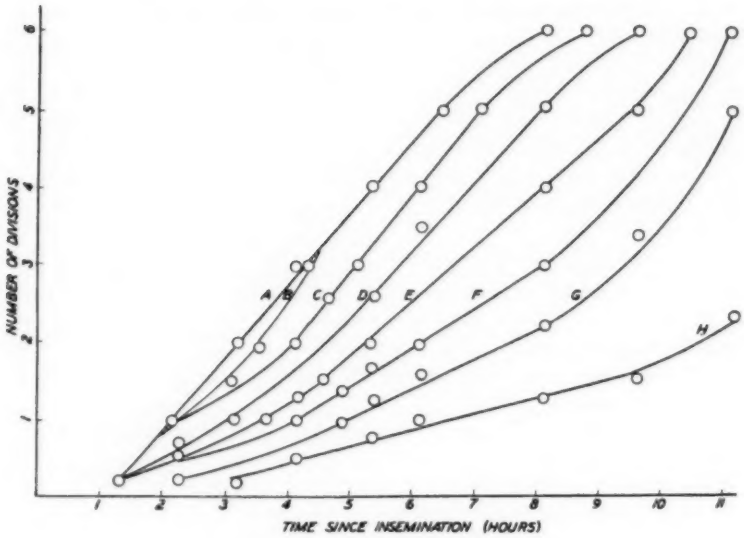

FIG. 1. Retardation of cleavage by radiation.

A. Control.
B. One-fourth of a second dosage.
C. One second dosage.
D. Two seconds dosage.
E. Four seconds dosage.
F. Eight seconds dosage.
G. Sixteen seconds dosage.
H. Thirty-two seconds dosage.

While many of the eggs irradiated for 4 or more seconds developed normally later, in most cases a retardation was observable even at later stages in development. Thus in Table I it will be noted that while 22 hours after insemination normal free-swimming blastulæ were formed from all eggs except those irradiated 4 seconds or more, those developed from eggs irradiated 4 and 8 seconds were still within

the fertilization membranes. Even 32 hours after insemination gastrulation in these eggs was just beginning when the controls and those given smaller doses had completed invagination. Ultimately all the above eggs, even those irradiated as long as 8 seconds, gave rise to gastrulæ normal to all appearances.

TABLE I

Later development of eggs irradiated ninety minutes after insemination at the dosages indicated

Dosage in seconds	22 hours after insemination	32 hours after insemination	46 hours after insemination
1/64, 1/32, 1/16, 1/8, 1/4 and control	Normal free-swimming blastulæ	Normal gastrulæ	Beginning of gut differentiation
1	Normal free-swimming blastulæ	Normal gastrulæ	Gut not yet differentiated
2	Normal free-swimming blastulæ	Normal gastrulæ	Gut not yet differentiated
4	Normal blastulæ but still within membranes	Blastulæ with beginning of invagination	Early gastrulæ
8	Normal blastulæ but still within membranes	Blastulæ with beginning of invagination	Early gastrulæ
16	Mostly abnormal motile balls. Some nonmotile blastulæ.	Mostly abnormal. Show delayed gastrulation.	As before
32	Coagulated cells	—	—
64	Coagulated cells	—	—

In some series (5 trials) of eggs irradiated 16 seconds a fair proportion of the eggs formed blastulæ and gastrulæ; in other series of eggs so irradiated cleavage resulted in a mass of motile cells which persisted without differentiation for as long as observations were made. After dosages of 32 seconds eggs developed into masses of undifferentiated cells which never became motile and early appeared opaque. Dosages of 64 seconds apparently inhibited even one division, though in some cases cleavage did occur; more often the attempt at cleavage was abortive, the apparent blastomeres failed to separate, often fused and then cytolized.

Differential Susceptibility of Eggs at Different Stages

It would be of interest to compare the susceptibility of unfertilized eggs, eggs just inseminated, eggs well after insemination, and eggs in the first cleavage. First of all, however, it was necessary to determine whether the unfertilized egg would become activated with the dosages used. Three series of 3 trials each were therefore made with 1/64, 1/16, 1/4, 1, 16, 64, and 256 seconds of irradiation. In no case was a

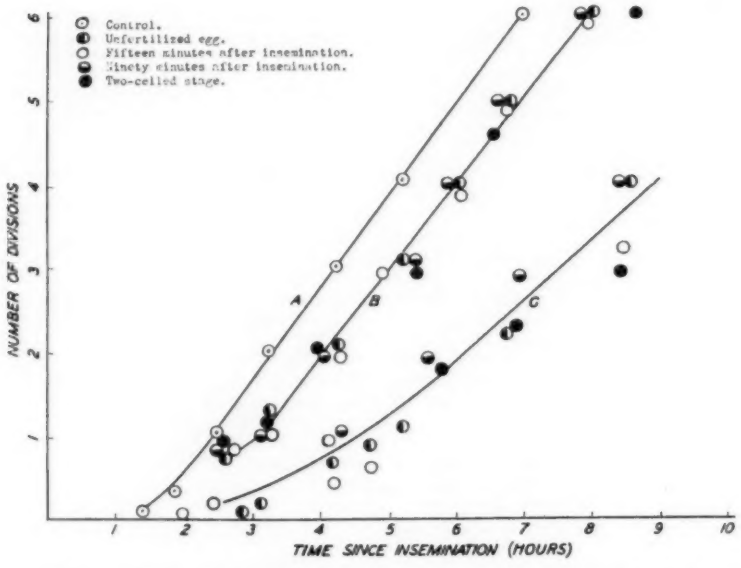

FIG. 2. Differential susceptibility to radiation of eggs at different stages of development.

A. Control.
B. One second dosage.
C. Sixteen seconds dosage.

normal fertilization membrane formed without insemination, nor was division apparent. The dosages used do not, then, activate the eggs to artificial parthenogenesis, but experiments showed that when the dosage was not too great, eggs so irradiated when inseminated would develop normally.

Three series of experiments (3 each) were now performed on eggs in the following stages: (1) unfertilized, (2) 15 minutes after insemina-

tion, (3) 90 minutes after insemination and (4) the two-celled stage with dosages of 1, 4, 16, and 64 seconds of irradiation. The results, excepting those for eggs irradiated 4 and 64 seconds, are given in Fig. 2. It is readily observed that there is no very great difference of susceptibility of these stages, one second of irradiation reducing the cleavage rate about equally in all cases (4 seconds reducing the rate still more, but omitted from the graph to avoid confusion), 16 seconds much more. From Fig. 2 it may be observed that there is a latent period before the effects appear. This is particularly so for all stages irradiated for one second and for the 2-celled stage irradiated at all dosages. The eggs irradiated 64 seconds gave but few abortive cleavages when irradiated before insemination or 15 minutes after insemination. When irradiated for 64 seconds, 90 minutes after insemination, most of the eggs passed into the 2-celled stage, and then cleaved abortively. Apparently at the time of irradiation the mechanism of cell division was already in full swing and could not be stopped. Of eggs irradiated for 64 seconds just after the first cleavage only a small proportion continued to divide and these only for a short time afterwards.

As in the previous experiments retardation was not only obvious during early cleavage but also at later stages, for when controls had developed into free-swimming blastulæ, some of the irradiated eggs had developed into non-motile blastulæ, and when controls had gastrulated, some of those irradiated had only begun to gastrulate. The data for later development are given in full in Table II.

Three sets of eggs in the 2-celled stages were irradiated with dosages equivalent to 1/4, 1/16, and 1/64 seconds, but in *no* case was the cleavage rate greater than that of the controls—in fact, there was a slight delay when the dosage of 1/4 second was given.

Effect upon the Medium

When the mercury-argon discharge lamp is in operation, ozone in readily detectable quantities is produced by the action of the short ultra-violet (1849A) on the oxygen of the air. To determine whether this was dissolving in the medium and causing retarded development in the experiments reported above, sea water was irradiated for 64 and 256 seconds, then eggs were added and the development compared with the controls (5 experiments, 256 seconds; 10 experiments, 64 seconds). Unfertilized eggs, eggs 15 and 90 minutes after insemination and 2-celled stages were used. In all cases there were no signs of retardation, cleavage in all cases being comparable to the controls. The retarded cleavage of eggs irradiated with the mercury-argon tube

is apparently due to the absorption of the ultra-violet radiation by the eggs and not to an effect upon the medium, and it is probably due practically entirely to radiation of λ2537A, since the only lines of

TABLE II

Later development of eggs irradiated at various stages in development

Dosage in seconds	Irradiated at fol. stage in development	21 hours after insemination	Percentage of blastulæ abnormal	27 hours after insemination	45 hours after insemination	No. of eggs used
1	Unfertilized egg	10% non-motile blastulæ	0.0	All motile	Normal gastrulæ	155
	15 min. after insemination	4% non-motile blastulæ	0.0	All motile	Normal gastrulæ	271
	90 min. after insemination	5% non-motile blastulæ	0.5	All motile	Normal gastrulæ	198
	2-celled stage	8% non-motile blastulæ	1.0	All motile	Normal gastrulæ, smaller than controls	188
4	Unfertilized egg	10% non-motile	0.9	All motile	Normal gastrulæ, smaller	346
	15 min. after insemination	77% non-motile	0.0	All motile	Normal gastrulæ	121
	90 min. after insemination	85% non-motile	11.6	All motile	Normal gastrulæ smaller than controls	104
	2-celled stage	99% non-motile	7.6	All motile	Normal gastrulæ, few incompletely invaginated	144
16	Unfertilized egg	All non-motile	0.0	Almost all non-motile	Some abnormal; most smaller than controls	250
	15 min. after insemination	All non-motile	13.7	20% motile	16% normal gastrulæ. Others abnormal morulæ	257
	90 min. after insemination	All non-motile	24.0	76% motile	87% gastrulate, small, somewhat abnormal	175
	2-celled stage	All non-motile	30.8	5% motile	5% normal gastrulæ, others abnormal morulæ	94
0	Controls	Motile blastulæ	0.4	Motile blastulæ	Gastrulæ	232

significant intensity in the mercury-argon tube spectrum are 3130, 3660, and several visible lines (4050, 4358, 5461, 5790A), all of which are relatively inert.

Experiments with Pure Light of λ2537A

To be certain that monochromatic light of λ2537A is the effective agent, a number of experiments were tried with pure light of this wavelength obtained from the monochromator already described. The unfertilized eggs were placed in a quartz cell and irradiated in the manner described in the previous paper (Giese and Leighton, 1935) usually for periods of 1, 4, 16, 64, 256 and 1,024 seconds. They were then trans-

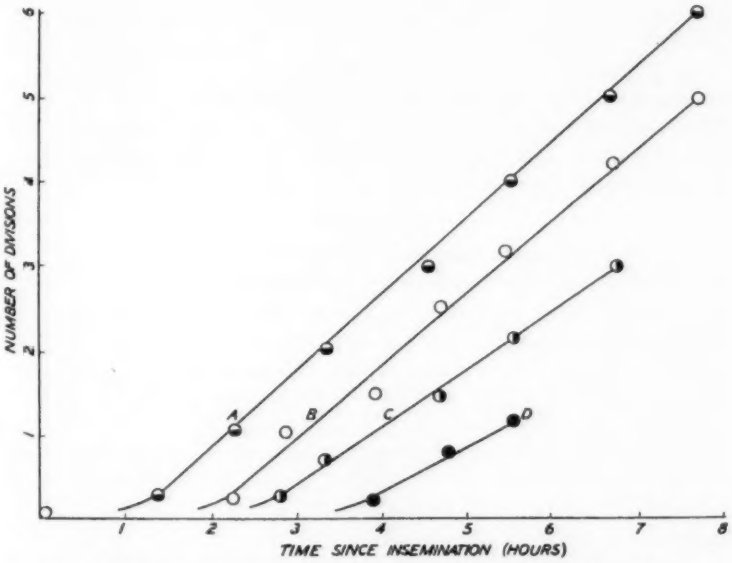

FIG. 3. Retardation of cleavage, λ 2537A; intensity, 9.74 ergs/mm.²/sec.
A. Control.
B. Sixty-four seconds exposure, extinction per egg: 2.91 ergs or 3.74×10^{11} quanta.
C. Two hundred and fifty-six seconds exposure, extinction per egg: 11.67 ergs or 1.50×10^{12} quanta.
D. One thousand and twenty-four seconds exposure, extinction per egg: 46.69 ergs or 6.00×10^{12} quanta.

ferred to watchglasses, inseminated and observed at hourly intervals until they had reached the 64-celled stage and then at 12-hour intervals until the gastrula stage. Seven series of experiments, the last three covering only the longer exposures, gave similar results and one series is plotted in Fig. 3.

It will be noted that retardation is evident when the dosage is

large enough. Smaller doses produced no noticeable retardation or acceleration. The results are in general similar to those already obtained with the mercury-argon discharge tube.

Extinction Measurements

It would be interesting to know the amount of energy which must be absorbed to produce the above effects. By interposing a cell first empty then full of eggs between the thermopile and the light source one can determine the fraction of the light incident upon the cell which is transmitted, I/I_0, where I is the intensity of the transmitted light and I_0 the intensity of the incident light. The fraction of the light extinguished, i.e., lost on passage through the cell can then be determined by subtracting the fraction transmitted from unity, $1 - I/I_0$. By determining the fraction of the area of the cell occupied by the eggs, A_e, one can determine the fraction of the light incident upon the eggs which is extinguished, $1 - I/I_0 \times 1/A_e$.

TABLE III

Data on extinction of light by sea urchin eggs

Exp.	No. eggs	A_e, fraction of area of cell bottom covered by eggs	$1 - I/I_0$ for λ's below:					$1 - I/I_0 \times \frac{1}{A_e}$ for λ's below:				
			2537A	4350A	5461A	5844A	6904A	2537A	4350A	5461A	5844A	6904A
1	3320	0.49	0.51	—	—	—	0.13	1.04	—	—	—	0.26
2	4431	0.65	0.63	0.38	0.27	0.23	0.14	0.97	0.58	0.42	0.35	0.22
3	3932	0.58	0.66	—	0.34	0.26	0.14	1.14	—	0.58	0.45	0.24
4	5964	0.88	—	—	—	0.38	0.25	—	—	—	0.43	0.28

A_e can be determined from the number of eggs, which can be counted, and the area of the egg effective in extinguishing light, which is approximately the area of a circle of the diameter of the egg. The average diameter of the egg used was the mean of the diameters of 50 eggs taken at random, $77.2 \pm 2.9\ \mu$ (two diameters were measured since almost one-half of the eggs were slightly ellipsoidal). A circle of this diameter has an area of $4{,}681\ \mu^2$. About 6,765 eggs would be necessary to completely cover the bottom of the cell used (area, $31.67 \times 10^6\ \mu^2$) with a layer one egg diameter thick. A_e is the number of eggs counted in a given experiment divided by 6,765.

The transmission of light of various wave-lengths by eggs was determined, the method used being similar in practically all respects to that described for *Paramecium* (Giese and Leighton, 1935), and the number of eggs was counted in each experiment. The experimental and derived data are recorded in Table III.

It is clear that practically all the light of λ2537A incident upon the eggs is extinguished, while at the longer wave-lengths a much smaller proportion is so lost. Part of the light extinguished is truly absorbed, part is lost by scattering from the surface of the egg and from the surfaces of small particles within the egg. Unfortunately it is very difficult to measure the scattering and it is impossible to obtain an approximation of the scattering as was done with *Paramecium* since unlike the latter the eggs absorb in the visible part of the spectrum as is quite obvious from the data in Table III. It is probable that as for *Paramecium* a considerable proportion of the light extinguished is actually scattered, possibly as much as 50 per cent of the total. However, the extinction measurements at least give the order of magnitude of the energy involved. The extinction by the eggs for the various experiments in Fig. 3 has been determined and the data have been added to that figure.

Discussion

The data of Fig. 3 are interesting because they give an idea of the number of quanta which must be absorbed to produce an effect. Thus eggs extinguishing between 1.57×10^9 and 2.54×10^{10} quanta, and probably absorbing about half this quantity, were not visibly affected and cleaved comparably to controls, only after a dosage of about 3.74×10^{11} quanta per egg was the rate of cleavage definitely retarded. Only doses short of those producing cytolysis stop cleavage for eggs extinguishing 1.24×10^{13} quanta went on developing as far as the 8-celled stage in many cases. Were one to assume that the average molecule in the egg protoplasm had a mass of the order of magnitude of the mass of the egg-albumin molecule, one would find the egg to possess some 2.5×10^{12} molecules. The actual number is probably much larger, but the figure indicates that a fair proportion of the molecules are affected or that certain molecules have absorbed many quanta before an effect is evident. The data are also indicative of the high power of recovery from injury possessed by the egg protoplasm.

From the data presented one may conclude that for the wave-length and the dosage series used, which covers the range usually employed in similar experiments, there is no evidence of acceleration of cell division. Following large doses of radiation there is retardation; following smaller doses the rate of cleavage is not noticeably different from controls.

These results do not, however, exclude the possibility of a stimulative effect of doses of ultra-violet light much weaker than here

employed. The radiations claimed by the Gurwitsch school of mitogenetic rays to be short ultra-violet and to be effective in increasing mitoses are postulated to be an entirely different order of magnitude from the radiations here used, in fact so weak as to defy physical detection. No attempt is here made to throw light upon this complex problem (see Rahn, 1936).

SUMMARY

1. There is a threshold dosage between 10^{10} and 10^{11} quanta below which radiation of λ2537A produces no observable change in the rate of cleavage. Beyond this threshold the degree of retardation increases with the dose.

2. Many of the retarded eggs develop normally, but are slower in reaching a given stage; others continue developing for only a short time, the degree of differentiation reached being inversely proportional to the dosage.

3. Unfertilized eggs, eggs 15 and 90 minutes after insemination, and eggs in the first cleavage do not exhibit strikingly different susceptibilities to the rays, although the later stages appear to be somewhat more susceptible.

4. The quantity of radiant energy which the eggs can absorb before being affected is quite large, as indicated by the extinction measurements reported, and serves as a rough measure of the power of repair of the egg protoplasm.

5. A series of dosages from a dose which cytolyzes to one which has no retarding effect upon cleavage with light of λ2537A failed to induce artificial parthenogenesis.

6. No evidences were obtained over the dosage series investigated for acceleration of the rate of cleavage.

BIBLIOGRAPHY

ALPATOV, W. W., AND O. K. NASTJUKOVA, 1933. The influence of different quantities of ultra-violet radiation on the division rate in Paramecium. *Protoplasma*, **18**: 281.

BOVIE, W. T., AND D. M. HUGHES, 1918. The effects of quartz ultra-violet light upon the rate of division of Paramecium caudatum. *Jour. Med. Res.*, **39**: 223.

CHASE, H. Y., 1937. The effect of ultra-violet light upon early development in eggs of Urechis caupo. *Biol. Bull.*, **72**: 377.

COBLENTZ, W. W., 1931. Sources of radiation and their physical characteristics: cold red ray and cold ultra-violet ray lamps. *Jour. Am. Med. Ass'n.*, **97**: 1965.

COBLENTZ, W. W., AND H. R. FULTON, 1924. A radiometric investigation of the germicidal action of ultra-violet radiation. *U. S. Bur. Stand. Sci. Paper* No. 495, **19**: 641.

GATES, F. L., 1929. A study of the bactericidal action of ultra violet light. I. The reaction to monochromatic radiations. *Jour. Gen. Physiol.*, **13**: 231.

GIESE, A. C., AND P. A. LEIGHTON, 1935. Quantitative studies on the photolethal effects of quartz ultra-violet radiation upon Paramecium. *Jour. Gen. Physiol.*, **18**: 557.

HERTEL, E., 1905. Ueber die Einwirkung von Lichtstrahlen auf den Zellteilungsprozess. *Zeitschr. allgem. Physiol.*, **5**: 535.

HINRICHS, M. A., 1928. Ultra-violet radiation and division in Paramecium caudatum. *Physiol. Zoöl.*, **1**: 394.

HUGHES, D. M., AND W. T. BOVIE, 1918. The effect of fluorite ultra-violet light on the rate of division of Paramecium caudatum. *Jour. Med. Res.*, **39**: 233.

HUTCHINSON, A. H., AND M. R. ASHTON, 1929. The specific effect of monochromatic light on the growth of Paramecium. *Can. Jour. Res.*, **1**: 292.

LEIGHTON, P. A., AND W. G. LEIGHTON, 1932. Some remarks on the use of thermopiles for the absolute measurement of radiant energy. *Jour. Phys. Chem.*, **36**: 1882.

LEIGHTON, W. G., AND P. A. LEIGHTON, 1935. Visual demonstration of the evaporation of mercury. *Jour. Chem. Ed.*, **12**: 139.

OSTER, R. H., 1934. Results of irradiating Saccharomyces with monochromatic ultra-violet light. I. Morphological and respiratory changes. *Jour. Gen. Physiol.*, **18**: 71.

RAHN, O., 1936. Invisible radiations of organisms. *Protoplasma Monographien*, vol. 9, Borntraeger, Berlin.

THE HISTOLOGY OF THE RETRACTOR MUSCLES OF THYONE BRIAREUS LESUEUR

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The histology of invertebrate muscle has received relatively little investigation and there exist at the present time marked gaps in our knowledge of this subject. A more critical study of invertebrate muscle might possibly yield clues of interesting phylogenetic significance. Recent research in the field of holothurian muscle physiology has also made desirable a more complete histological study of these muscles.

The only extensive paper on the histology of holothurian muscle (Hall, 1927, on *Cucumaria*) completely misinterprets the essential structure and has been responsible for a number of erroneous conclusions by workers in muscle physiology.

This paper concerns itself with the more salient features of the histology of *Thyone* retractor muscle. The interpretations are opposed to the conclusions of Hall on the strikingly similar muscles of *Cucumaria*.

Materials and Methods

The retractor muscles of *Thyone* occur in five main bands which run from the lantern to points of attachment on the five longitudinal muscles of the body. The individual bands may be single or broken up into a number of divisions. They are extremely extensible and in adult specimens may vary in length from about 5 mm. in the contracted condition to 5 or 6 cm. in normal extension. Except for the two regions of attachment the muscles are free of the tissue and are easily removed.

The muscles were fixed in varying degrees of contraction in Bouin's or Helly's fixing fluids. In order to obtain normal extension it was necessary to immerse them for some time prior to fixation in 0.3 molar magnesium chloride solution. The muscles were dehydrated in alcohol, cleared in xylene, and imbedded in paraffin. Sections were cut at 5 and 10 micra.

Heidenhain's iron hæmatoxylin proved to be the most useful stain for the muscle fibers. Mallory's aniline blue and Dominici's

stain were most favorable for the details of the connective tissue network.

Attempts were made to macerate the muscles in Bouin's fluid for periods up to three weeks and in varying strengths of nitric acid. These techniques, however, did not prove to be very satisfactory.

Observations

Microscopic examination of the fresh muscle reveals extremely long fibers grouped into indistinct bundles. In cross-sections of fixed and stained muscles this bundle arrangement is clearly discernible (Figs. 1 and 4). The inner portion of the muscle consists of hemicylindrical bundles containing from two to fifteen fibers which tend to be arranged about the periphery of each bundle. The outer portion of the muscle exhibits no definite bundles. Longitudinal sections reveal that the bundles do not maintain the same complement of fibers throughout their length. Fibers pass from one to the other of adjacent bundles and in the periphery of the muscle there is no definite association of fibers.

The spaces between the bundles and the fibers of the bundles are occupied by a delicate reticulum of connective tissue which is markedly condensed about each bundle (Figs. 1 and 4). The connective tissue appears to have the nature of a lattice-like framework with the thickest fibers running perpendicular to the muscle fibers. In sections of contracted muscle the elongated connective tissue nuclei are also seen to lie with their long axes in this direction. The connective tissue fibers are especially coarse and dense in the periphery of the muscle.

Connective tissue and tissue space constitute a relatively large proportion of the muscle in contrast to mammalian smooth muscle and some invertebrate muscle, e.g. retractor muscle of *Phascolosoma*. Steinbach (1937), in his studies on potassium and chloride in *Thyone* muscle, has estimated the volumes occupied by the muscle elements and tissue space. According to Steinbach, chloride diffuses freely into 43 per cent of the muscle—a figure which by his interpretation corresponds rather closely to the extrafibrillar space (tissue space and sarcoplasm). However, since Steinbach tentatively accepted the histological conclusions of Hall, his results may be reinterpreted. The structures which Steinbach, following Hall, interpreted as fibrils are actually fibers. Those structures interpreted as fibers are bundles of fibers. Thus it is found that chloride space corresponds closely to tissue space (47 per cent of *Thyone* muscle) as has commonly been believed (see Fenn, 1936), and does not include the sarcoplasm of the muscle fibers.

Fischer (1937) found that *Thyone* retractor muscle exhibited a birefringence of 60 per cent of that of *Phascolosoma* retractor muscle. (Histologically, *Phascolosoma* retractor muscle exhibits densely packed elements.) Since birefringence may be presumed to be associated with contractile elements, Fischer's figure suggests that these elements are fewer in number than in *Phascolosoma* muscle, and it may be further interpreted as lending support to the figure given above for tissue and chloride space in *Thyone* muscle.

As far as it has been possible to determine, the fibers are uninucleate. The nuclei occupy lateral positions on the fibers (Figs. 1 and 3), only rarely are they found within them (Fig. 4). In the contracted fiber the nuclei are rounded and are usually found to occupy a typical lateral position on the fiber. With the proper staining technique a layer of faintly staining cytoplasm may be seen to surround the nuclei. Often a thin strand of this cytoplasm forms the only connection between the nucleus and the muscle fiber. In the extended muscle the nuclei are elongated in the axes of the fibers and lie closely applied to them.

The appearance of the fibers in cross-section is somewhat variable. In the contracted condition they tend to be circular or semicircular (Fig. 1). Occasional fibers are polygonal. The stretched fibers may be circular or oval in transverse section, but are more often flattened or angular (Fig. 4). The fiber diameter varies from 5–10 μ in contraction to 2–4 μ in extension.

Teased preparations of muscle reveal the spindle-shaped character of the fibers. The tips of the fibers are long and tapering in extension (Fig. 5), bluntly rounded in contraction.

It was extremely difficult to determine the lengths of the muscle fibers since it was almost impossible to isolate these elements. The closely investing connective tissue binds the fibers securely together and all attempts at maceration and teasing were largely unsuccessful. Careful study of the best of these preparations, however, revealed that the fibers do not extend the whole length of the muscle. At

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Explanation of Figures

All drawings were made with the aid of a camera lucida at a magnification of × 745.

FIG. 1. Cross-sections of two bundles of fibers from a contracted muscle.
FIG. 2. Portion of a longitudinal section of an extended muscle.
FIG. 3. Portion of a longitudinal section of a contracted muscle.
FIG. 4. Cross-sections of two bundles of fibers from an extended muscle.
FIG. 5. Fragment of a teased muscle.

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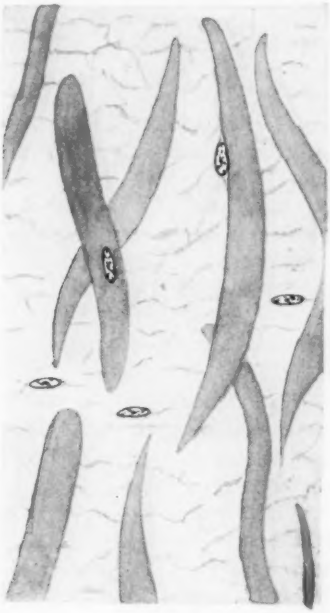

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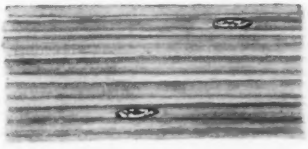

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PLATE I

various points along a teased muscle natural unbroken tapering fiber terminations could be found (Fig. 5).

Discussion

The contention of Hall (1927) that *Cucumaria* muscle consists of large fibers containing a few giant fibrils forms an untenable theory. From Hall's figures it is apparent that the histological picture of *Cucumaria* muscle is almost identical with that of *Thyone*. In *Thyone* muscle, however, the nucleated condition of the so-called fibrils is clearly evident. It must be concluded, therefore, that these are muscle fibers and not fibrils. It is true that the nuclei often have a tenuous attachment to the fibers, but this attachment is nevertheless definite, and, as previously stated, nuclei are occasionally found within the fibers. The free nuclei within the bundles, which Hall interpreted as muscle nuclei, are clearly constituents of the connective tissue. Further, the failure of the peripheral elements to be arranged into bundles (muscle fibers of Hall) does not support Hall's conclusion that these are fibrils.

The conception that the muscle fibers are long spindle-shaped elements is supported by the studies of Gerould (1896) on *Caudina* and Jordan (1914) on *Holothuria* and *Stichopus*. According to Gerould, muscle fiber nuclei in *Caudina* occupy a lateral position similar to those in *Thyone*.

du Buy (1936), studying the sensitivity of *Thyone* muscle to certain drugs, found that these muscles exhibited a physiological condition intermediate between vertebrate smooth and vertebrate striated muscle. Microscopically they resemble vertebrate smooth muscle. No histological similarity with vertebrate striated muscle can be detected with the possible exception of their extreme length. The fibers are homogeneous, devoid of striations or fibrillar structure.

Summary

The retractor muscles of *Thyone* consist of long spindle-shaped smooth muscle fibers. The fibers appear to be uninucleate. The nuclei usually occupy a lateral position and are attached to the fiber by a delicate layer of cytoplasm. Occasional nuclei are found completely within the fibers.

In the center of the muscle the fibers occur in bundles of two to fifteen. No such organization is exhibited in the periphery of the muscle.

The fibers do not remain associated with a single bundle throughout their length but pass freely from one bundle to the other.

Measurements of fiber length were not obtained, but it is evident from study of teased preparations that they do not extend the full length of the muscle. Fiber diameter varies from 5–10 micra in contraction to 2–4 micra in extension.

The connective tissue forms a lattice-like network which is concentrated about each bundle and in the periphery of the muscle. The heaviest fibers of the reticulum tend to run perpendicular to the axes of the muscle fibers.

LITERATURE CITED

DU BUY, H. G., 1936. Separation of the conducting and contractile elements in the retractor muscle of Thyone briareus. *Biol. Bull.*, **71**: 408.

FENN, W. O., 1936. Electrolytes in muscle. *Physiol. Rev.*, **16**: 450.

FISCHER, E., 1937. The influence of length, tension and tone upon the birefringence of smooth muscles (Phascolosoma and Thyone). *Biol. Bull.*, **73**: 363.

GEROULD, J. H., 1896. The anatomy and histology of Caudina arenata Gould. *Harvard Bull. Mus. Comp. Zoöl.*, **29**: 123.

HALL, A. R., 1927. Histology of the retractor muscle of Cucumaria miniata. *Publ. Puget Sound Biol. Sta.*, **5**: 205.

JORDAN, H., 1914. Über "reflexarme" Tiere. IV. *Zool. Jahrb.*, **34**: 365.

STEINBACH, H. B., 1937. Potassium and chloride in Thyone muscle. *J. Cell. and Comp. Physiol.*, **9**: 429.

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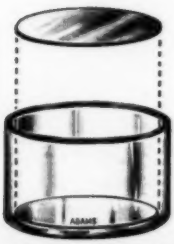

CLAFF RECOVERY DISH

See article on page 178 of this issue by George W. Kidder and C. Lloyd Claff, "Cytological Investigations of Colpoda cucullus."

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